2020-01-01

Comparative Experimental And Theoretical Study Of Dopamine And Serotonin Interaction

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COMPARATIVE EXPERIMENTAL AND THEORETICAL STUDY OF DOPAMINE AND SEROTONIN INTERACTION

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To my

Mother, Isela, and Father, Mario

With love

COMPARATIVE EXPERIMENTAL AND THEORETICAL STUDY OF DOPAMINE AND SEROTONIN INTERACTION

BY

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THESIS

Presented to the Faculty of the Graduate School of

The University of Texas at El Paso

in Partial Fulfillment

of the Requirements

for the Degree of

MASTER OF SCIENCE

Department of Physics
THE UNIVERSITY OF TEXAS AT EL PASO
May 2020

ACKNOWLEDGMENTS

Foremost, I would like to express my deep appreciation to my advisor, Dr. Felica S. Manciu of the Physics Department at The University of Texas at El Paso, for her immense knowledge, encouragement, and most importantly patience and kindness. She has always been supportive and understanding in any aspect of my graduate education and research. Dr. Manciu has always believed in my skill as a student even when I would doubt myself. Her trust in me helped me to be more confident and believe in my abilities.

Besides my advisor, I would like to thank the rest of the thesis committee: Dr. Binata Joddar, Dr. Deidra Hodges, and Dr. Marian Manciu for their support, suggestions, and encouragement.

Finally, I would like to thank my family: my parents Isela and Mario for always believing in me and supporting me in everything. You have worked very hard to allow me to have the opportunity to accomplish my dreams and I will always be grateful for that.

ABSTRACT

To accurately identify and measure the concentrations of dopamine (DA) and serotonin (5-HT) in mixtures of these neurotransmitters without labeling, a comprehensive, comparative computational and Raman experimental analysis is provided. While the distinction between these two analytes may be accomplished for concentrations in the millimolar range of these mixtures, their accurate quantification remains unattainable. As shown for the first time in this study, potential creation of a new composite resulting from their interactions with each other could be a reason for this lack of quantification.

Although this new hydrogen-bonded complex greatly complicates future analyte differentiation and quantification at concentrations typical of clinical thresholds (i.e. nanomolar concentrations), it may also create new opportunities for use in drug distribution and pharmaceutical investigations. This remark not only is based on the chemical interactions studied here by both theoretical and experimental viewpoints but also on the biological proximity of these neurotransmitters. This work provides a significant, complementary contribution to a deeper understanding of neuronal processes and potential future creation of label-free biosensors.

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CHAPTER 1
INTRODUCTION

1.1 Neurotransmitters

The brain is composed of billions of nerves that are continuously communicating and sending signals with one another controlling what the body does, feels, and thinks, making the brain the most complex organ in the human body. The human brain contains 100 billion neurons that can communicate with other cells through relative long distances and relay the nervous system messages to muscles and other cells, but most of the neurons communicate with each other to regulate behavior [1]. At the end of each neuron's cell body is a collection of fibers called dendrites, and on the other side of the cell is the axon, which is responsible for sending signals in the form of electrical impulses[2]. Neurotransmitters are chemicals produced in the nerve fibers and work as chemical messengers to assist signaling between neurons in the brain.

Neurotransmitters are the molecules used by the nervous system to transmit information between the neurons, then, to the muscles. Information between two neurons happens at the synaptic cleft, a small gap between the synapses (Figure 1). The neurotransmitters move across the synapse gap to other neurons. The neurotransmitters affect the neurons, either excitatory, inhibitory, or modulatory. An excitatory transmitter can generate an electrical signal called an *action potential*, which is received by a neuron. On the other hand, an inhibitory transmitter prevents this process from happening, depending on the binding receptor [3]. Any malfunction in the production of neurotransmitters can result in neurological diseases. Consequently, neurotransmitters are becoming an increasingly interesting topic in the diagnosis of neurological

disorders, with each neurotransmitter becoming medically helpful for the specific cause of the disease.

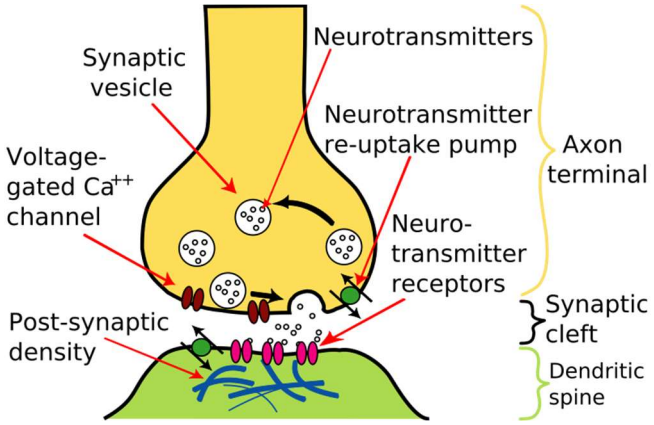

Figure 1. The neuron gap, synaptic transmission, and neurotransmitters. In R. M. Julien[4]

1.2 Dopamine and Serotonin

Two of the most important neurotransmitters produced and present in the central nervous system are serotonin (5-hydroxytryptamine or 5-HT) and Dopamine (DA). These two neurotransmitters are also most known as "happy chemicals." When a person is doing any activity that he/she enjoys, dopamine and serotonin are released, thus, being the chemical that is responsible for such feelings.

Dopamine functions have a motivational component of reward-motivated behavior, of regulatory movement, attention, addiction, and learning [5]. DA is synthesized mainly in central regions of the brain, for example, the substantia nigra pars compacta (SNpc), the ventral tegmental area (VTA) and the arcuate nucleus of the hypothalamus[6]. Since DA is known to contribute to the feeling of pleasure and satisfaction, it is the neurotransmitter responsible for addiction and depression. DA is essential in the motor system; when the metabolism failed to

produce enough of it, Parkinson's disease can result. On the contrary, the overproduction of dopamine can cause schizophrenia and other severe mental disorders. 5-HT helps regulate mood and social behavior, appetite and digestion, sleep, aggression, memory, and sexual desire. Overproduction of 5-HT is also linked to depression and anxiety, although it is unclear if depression causes the reported low levels of serotonin [7]. 5-HT is found primarily in the central nervous system, digestive system, and blood platelets. It is also thought to be active in the construction of smooth muscle. Low 5-HT may cause poor memory, low mood, anxiety, and difficulty sleeping since it is a precursor for melatonin, which helps regulate the body's sleep-wake cycles and the internal clock. Serotonergic innervations can be found through cell bodies lying in the Dorsal (DRN) or Median (MRN) raphe nucleus, and lower brainstem regions in a column of the raphe, connecting to the spinal cord and all divisions of the brain [8]. Several comprehensive studies of DA and 5-HT have been completed for both neurotransmitters, and progress has been made in understanding their brain functions. However, there are still many studies to be done on their interactions and complicated relationship, from both neurological and chemical perspectives. The understanding of these neurotransmitters' biochemical effects is crucial for the administration of drugs; regarding the efficiency of neurological treatments for such phycological disorders.

Many dopamine-focused treatments have been shown to significantly help with depression and mood stabilization problems, such as dopamine precursor *Levodopa* and monoamine pharmaceutical *Pramipexole*. These treatments can also help with bipolar depression and other major depressive disorders. Observing DA current drug treatments suggests that its regulation has an interaction with the pathogenesis of behavioral and mood disorders, possibly in conjunction with 5-HT mechanisms and other catecholamine analytes[6]. In this context, it is

also known that DA and 5-HT compete with one another, with the latter eventually serving as a potent modulator; 5-HT affects behavior, decision-making problems, both normal and abnormal. Despite the opponency and different functions of DA and 5-HT, they both coexist in a close relationship.

1.3 Pathways for DA and 5-HT

DA and 5-HT are primarily centered in the midbrain; the dopaminergic system is having the VTA and the nigrostriatal as its primary pathways and the 5-HT having the dorsal and medial raphe nuclei as its central pathway as seen in the Figure 2. [6] The interaction between DA and 5-HT is expected because the DRN contain cortex and striatal regions projected with the median raphe nuclei entering the limbic areas; thus, revealing the proximity of these neurotransmitters.

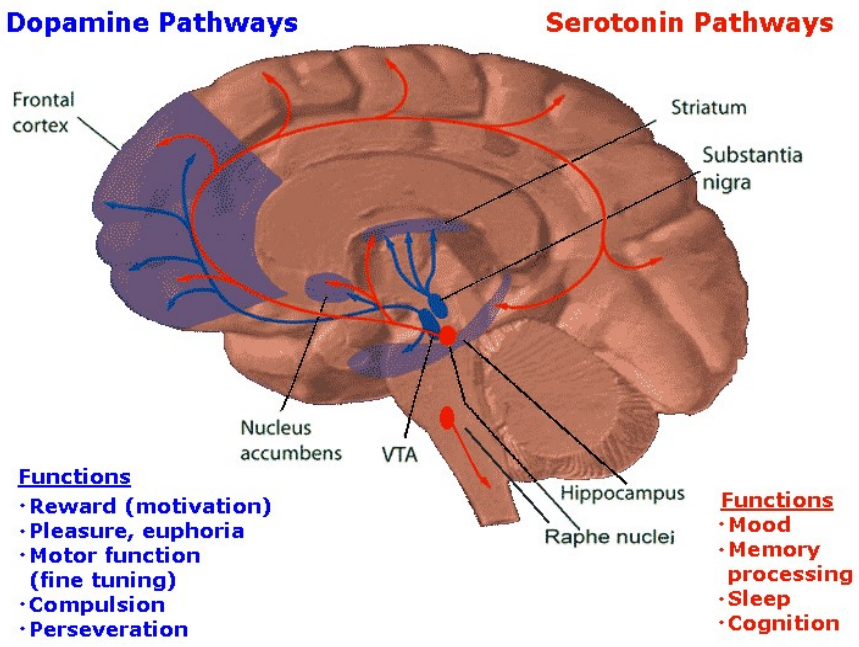

Figure 2. Dopamine and Serotonin Pathways and function in the brain. [9]

Therefore, the striatum ultrastructure analysis supports 5-HT inhibitory effects on dopaminergic neurons in the midbrain and the forebrain by the existence of 5-HT physical neuronal terminals in extremely closed vicinity to those of DA neurons. For example, it has been observed that 5-HT has inhibitory regulatory effects on DA when lesions happen intentionally, interrupting the dorsal raphe-nigra pathway and leading to systematic disruption of the inhibitory modulation of the DA network[6]. Another indicator that DA and 5-HT interact with one another, aside from neuronal inhibition of DA, consists of their chemical analysis.

Both DA and 5-HT have structural features such as hydroxyl groups that are suitable model compounds for studying intermolecular interactions among themselves and with other proteins. At physiological levels, while theoretical models were constructed and reported for their interactions, correspondence between these models and experimental data through optical methods has not yet been established. A significant obstacle is that most of the analytes have been tagged with a variety of fluorescent dyes or attached to different nanoparticles, making it difficult to measure with optical techniques. Although labeling the neurotransmitters helps with the detection by optical methods, it also prevents measuring the interaction between the analytes and the neurotransmitters and changes the entire chemistry and dynamic process. It is a challenge to measure them label-free simultaneously. On the other hand, it is desired for computationally and experimentally comparison perspectives and for precisely depicting their interactions.

Surface-Enhanced Raman Spectroscopy (SERS) is the perfect technique for such measurements, as it is known to be a sensitive method for label-free detection of analytes at molecular levels. SERS has been known to be able to measure independent analytes, but there still are no reports of label-free, simultaneous detection of multiple neurotransmitters at phycological levels[6]. Having attached them with different types of fluorescence dyes or tagged

them with various nanoparticles, while indeed succeeded in examining these samples, also encountered the multiplex problem. Another drawback is that labeling neurotransmitters prevent the ability to monitor their interactions with other analytes or neurotransmitters; therefore, changing the overall chemistry of the sample. Only through label-free, simultaneous detection can the desired results of computational and experimental comparisons be obtained, and the sample adequately evaluated; however, this is a challenging task.

Another impediment for simultaneous measurements and label-free detection of neurotransmitters at physiological levels using SERS is the fact that at the metallic SERS surfaces, chemical interactions arise, changing the vibrational lines of analytes under study; making shifts in frequencies and changes in intensities of neurotransmitter's vibrational lines. Sometimes, these changes can be so dramatic to result even in the complete disappearance of the vibrational lines.

An example is the DA vibrations at 750 and 795cm^{-1} that were either associated with in-plane phenolic ring bending mode or with the out-plane O-H and C-H bending modes, which completely disappeared in SERS measurements[6]. The similarity between DA and 5-HT chemical structures is also a problem, as it induces close overlapping of dominant characteristic signatures of neurotransmitters in SERS data, complicating their accurate reading and simultaneous label-free detection. An example is the vibrations at 1170, 1275, 1310, 1520, and 1620 cm^{-1} at low concentrations of DA and the characteristics features around 1140, 1235, 1350, and 1550 cm^{-1} for serotonin.[6] If we consider a potential (+-) 5 cm^{-1} error in such measurements of frequencies, it undermines the accuracy of data obtained from their simultaneous detection at physiological levels. The frequencies used to distinguish between the neurotransmitters are the nonoverlapping signatures of 1290 cm^{-1} for DA and the 1550 cm^{-1} for 5-HT. At high

concentrations of DA and 5-HT, there is no need to use SERS; Simultaneous detection is possible and reported[10]. Theoretical analysis has been taken on to separate DA and 5-HT, and their potential interactions to overcome limitations. Furthermore, analysis of the interaction between these two amines in the brain, in normal and pathological conditions, can provide new pharmacological advances for the treatment of several neuropsychiatric disorders. The results of these interactions between DA and 5-HT are thus of tremendous importance and interest for a scientist working in the field of neuroscience advances.

CHAPTER 2

EXPERIMENTAL BACKGROUND AND METHODOLOGY

2.1 Spectroscopic Techniques

Spectroscopy is the branch of science used to investigate and measure spectra produced when matter interacts with or emits electromagnetic radiation, including electrons, protons, and ions. It is also the interaction with other parties as a function of collision energy. Raman spectroscopy, which is the technique employed in the current study, was named after its inventor Sir Chandrasekhara Venkata Raman, an Indian Physicist. C. V. Raman discovered that the interaction of incident radiation with a molecule produces radiation with different frequencies. The light from a laser (i.e., incident light radiation) is radiated by scattering. The occurring changes in frequencies contribute to identifying any material, being a “fingerprint” for a molecule; no two molecules vibrate the same. Spectroscopy methods are based on the phenomena of emission, absorption, fluorescence, and scattering that can be used to obtain a qualitative and quantitative analysis of molecules in the sample. [11] The qualitative analysis establishes the identity of constituents in the sample, and quantitative analysis estimates the concentration of a constituent/chemical substance in the sample being tested. The energetic modifications of molecules are made of translational, rotational, vibrational, and partly electrical types; these are the degrees of freedom of the sample's bonds and can change as the molecule is radiated with a monochromatic laser.

The electric energy gives rise to absorption and emission in the ultraviolet and visible regions of the electromagnetic spectrum. Most of the scattered radiation is elastically scattered, having the same frequency as the incident radiation. This radiation is called Rayleigh Scattering.

Only a small amount of the radiation has a different frequency than that of the incident radiation, constituting the Raman scattering/effect, which is employed in current data collection. No change in the wavelength of the individual photons occurs in Rayleigh scattering. Spectroscopic techniques such as Raman and Fourier transform infrared (FTIR) absorption can be used to study the composition of molecules in samples. Fourier spectroscopy, a general term that describes the analysis of varying signals into its constituent frequency components. Mathematical approaches named after J.B.J. Fourier are potent in spectroscopy and have been discussed in detail elsewhere [12]. FTIR can be applied to a variety of research fields like infrared absorption, Raman, nuclear magnetic resonance (NMR), and electron spin resonance (ESR).

2.2 Raman Spectroscopy

As previously mentioned, Raman Spectroscopy is a scattering technique based on the Raman effect or Raman scattering. It is used in the scientific analysis of materials to provide chemical and structural information of various compounds. The samples are illuminated with an intense source of monochromatic laser consisting of the incident radiation and interact with the molecules in the sample. This incident light radiation produces the scattering radiation, which is latter analyzed from data collection. When the incident radiation has a more significant energetic (frequency) value than that of the scattered radiation, Stokes frequency lines appear in the Raman spectrum. Anti-tokes lines appear when the incident frequency is lower than the initial frequency. These Stokes and anti-Stokes vibrations are either above or below the Rayleigh scattering line, which happens when the radiated frequency matches the initial one (Figure 3).

This terminology arose from Stokes's rule of fluorescence, which stated that fluorescent radiation always occurs at lower frequencies than that of the exciting radiation. [11]

Pure rotation gives a spectrum in the microwave region or far-infrared, and vibrations of molecules provide a spectrum within most of the infrared spectral region. The radiation scattered by the sample is latter recorded at the spectrometer or CCD camera and further analyzed. When measuring aqueous solutions, because water is a weak scattered in the VIS range, no unique accessories are required for such Raman measurements. Concerning temperature-dependent Raman measurements, both Stokes and anti-stokes lines scattering are frequently used; the ratio of the same Raman peak intensities are calculated [13].

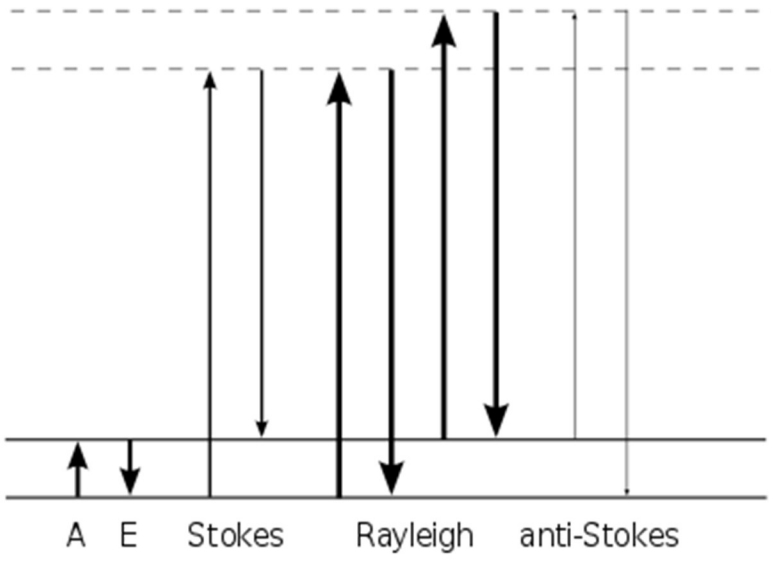

Figure 3. Raman stokes and inti-stokes and Rayleigh scattering processes via virtual states (denoted by dash lines). On the left, absorption A and emission E between real energy levels. [14]

In general, the Stokes lines are enough to determine the chemical structure of the sample. However, due to the fluorescence of reflective materials, which can hide part of fully the vibrational Raman spectrum (photoluminescence being the first-order optical scattering), other more sophisticated instruments are considered, as well as the appropriate excitation wavelength. Since this limitation in Raman spectroscopic measurements does not occur in infrared investigations, the sensitivity of the latter technique can be higher. On the other hand, FTIR spectroscopic method is sensitive to water absorption, making it inappropriate in studying aqueous materials. The Raman effect is weak, typically 10^{-8} of the intensity of the incident, exciting radiation. The classical approach of the Raman effect takes into account the scattering of the molecules as a collection of atoms undergoing simple harmonic oscillations, considering the quantization of the vibrational energy [15]. The electromagnetic radiation consists of its wavelength λ, the frequency ν, and its wavenumber $\tilde{\nu}$. The wavenumber is measured in cm^{-1}, which is the number of waves in one wave train

$$\tilde{\nu} = \frac{\nu}{c} \qquad \tilde{\nu} = \frac{1}{\lambda} \tag{2.1}$$

Where c = $2.99792458 \times 10^{10}$ cm/s is the speed of light, v is the frequency in the cycles per second or Hertz, and λ is the wavelength in cm. The wavelength is a property of radiation not of molecules; the only properties that unite radiation and molecules are the energy and frequencies. In quantum theory, the energy of the photon, E_p, that is given by:

$$E = h\nu \qquad E = hc\bar{\nu} \tag{2.2}$$

where h=6.6256×10^{-27} erg-sec is plank constant. The photon can be absorbed or emitted by the molecule. When this event happens, the rotational, vibrational, or electrical energy of the molecule changes due to the conservation of energy. The energies are positive if absorbed and

negative when lost. The radiation causes an induced dipole moment in the sample, which is related to the polarizability, the dipole moment P, and the electric field E, by:

$$P=\alpha E \qquad (2.3)$$

The dipole moment is a vector and the polarization a tensor. The polarization measures the deformability of the electron cloud of the molecule by the electric field. This change in the electron cloud allows for optical frequencies to undergo amplitude modulation of the oscillations of the dipole moment, allowing for measurable Raman frequency components. A Raman spectrum is a plot of Raman scattering intensities as a function of change in frequency from the incident frequency, also called Raman shift.

Since most of the samples are measured at room temperature or in thermodynamic equilibrium, the molecules are in their ground states. At room temperature, the Stokes lines are more intense than the anti-Stokes lines; an observation that can be exploited to examine the sample as their ratio depends on temperature. [11] The Raman effect is a different form of fluorescence. The fluorescence of the incident radiation is completely absorbed, making the molecules to be in an excited state. The emission of the scattered light can be the same as that of the incident radiation (elastic scattering) or at different frequencies (Stokes and anti-Stokes). The significant difference between fluorescence and the Raman scattering is that the latter effect can happen for any frequency of incident radiation; it is not a resonant effect. In practice, this means that the fluorescence effect is anchored at specific frequencies only. On the other hand, Raman scattering has a constant separation from the excitation frequency. During molecule vibration, it is an essential requirement for the polarization to change to obtain a Raman spectrum. Also, it is vital to avoid fluorescence in the sample, since fluorescence is much stronger than the Raman scattering, interfering with the gathered results. An objective lens collects the electromagnetic

radiation that is emitted from the spot that's been targeted with a laser and further sent to a monochromator, which transmits a mechanically selectable narrow band of wavelengths or radiation from a range of wavelengths available at the input.

2.3 Surface-enhanced Raman spectroscopy (SERS)

Surface-Enhanced Raman spectroscopy (SERS) has been used numerous times in literature for the accurate detection of analytes [16]. SERS is a powerful spectroscopic technique that by amplifying the electromagnetic field allows for highly sensitive structural detection of low concentrations of analytes. Many methods have been used to increase the Raman scattering signal, but SERS is one method that has been showing significant signal improvement. As such, small amounts of molecules and the single molecule can now be optically characterized in detail. SERS experimentation needs to take into consideration the sample and optical set-up, to effectively obtain the high-quality signal and identify the chemical structure of these small groups of molecules. It is essential to mention that the maximum SERS enhancing region decreases extremely rapidly with distance from the objective; the most significant enhancements are found in the few nanometers closest to the substrate surface[17].

Because of SER's high sensitivity in detecting biological samples, it has also been proposed for use in the development of biosensors. SERS employment in biosensors is a vast topic, ranging from the detection of many diseases, such as cancers, Alzheimer's disease, and Parkinson's disease. Metallic nanoparticles, also called plasmonic materials, are known to be more prompt to the electromagnetic radiation effect on their electron density cloud in the sample. SERS is a highly effective vibrational spectroscopy that allows label-free, highly sensitive, and

selective detection of analytes by the amplification of localized electric fields on the surface of the plasmonic materials radiated with the monochromatic light. Nobel metals such as Silver (Ag) and Gold (Au) are used as plasmonic nanoparticles to enhance the electric fields generated when the conduction band electrons in the metal nanoparticles smaller than the wavelength of the exciting light couple with the surface polaritons and oscillate with a frequency referred to as the localized surface plasmon resonance (LSPR). [18] The main optical property of the SERS substrate is the LSPR. The excitation of SERS metallic substrates by the laser produces collective electrons oscillations inside the evanescent nanoparticle wave. Oscillations of electrons in the plasmonic material cause the LSPR effect, which is involved in the enhancement of the electromagnetic field.

SERS methods of investigations can be classified into two categories, direct and indirect. Direct sensing depends on the absorption of an analyte molecule to a plasmonic substrate, and the resulting signal corresponds to the vibrational modes of the analyte. In indirect sensing, the SERS signal is obtained from a reporter molecule, a dye or molecule that emits strong Raman scattering, instead of the analyte. For direct sensing, it can be challenging to connect spectral intensity to the concentration because the signal is non-linear at high levels of the analyte due to high spacing between particles, which causes weak enhancement. In biofluids, the direct sensing results in spectra that are difficult to interpret to get high-quality results, difficulty coming from the enhancement of the components of the fluid itself [18]. Thus, to analyze such biological samples, the sensing techniques must be sensitive enough to measure the small amounts of the sample.

The excellent sensitivity of the SERS makes it is a fundamental technique to obtain results from such analytes. It has been shown that SERS can receive single-molecule limits of

detection (LODs) in the optimal case of a molecule with an electronic transition near the frequency of the LSPR (resonant), a broad Raman cross-section, and an attractive electrostatic interaction between the analyte molecule and the substrate.[18] In order to sufficiently measure neurotransmitters, which are the analytes of interest in the current research, the sample needs to be extremely close to the plasmonic materials. In this way, the Raman scattering signal can be amplified. Also, the plasmonic material must be of "rough surface" for the sample to be trapped in place when measuring. The enhancement of the Raman signal depends on the shape and the size of the nanostructure. Understanding the changes in concentrations of neurotransmitters produced in some areas of the brain is one of many applications of SERS that can help the diagnosis of neurological diseases. In the current work, we are planning to use SERS for the simultaneous detection of dopamine and serotonin.

2.4 Experimental set-up – the 300RAS alpha WITec system

While measurements using Raman spectroscopy can be performed easier in comparison with other methods, it does not give a controlled sampling volume. Confocal Raman microscopy is the ability to analyze the desired amount of the sample in the x-axis and y-axis (lateral), as well as in the z-axis (depth) of the sample. The ideal microscope will examine each point of the sample at a time by measuring the scattered and absorbed radiation. However, to achieve a quality local spectrum, the amount of scattered light should be small; this can be done by setting a pinhole aperture in front of the laser. When hitting a small spot instead of the whole sample, the amount of the scattered light is reduced without affecting the focal brightness. The second pinhole aperture that is placed between the microscope objective and the image plane rejects the

remaining scattered rays originated from any out-of-focus points on the sample and functions as a spatial filter.[19] While the first pinhole reduces scattered light and improves the image quality, the second aperture eliminates the imager degrading, allowing for bulk specimens to be analyzed in optical sections by controlling the depth of the field.

Clear advantages of confocal Raman microscopy use happened in many fields of nanoscience, material science, and nanotechnology. Another rapidly growing application of confocal Raman is a bio-science arena and pharmaceutical industry. The ability to give improved axial and spatial resolution allows us to get a detailed examination of cells in their natural state. Very little sample preparation is needed for such measurements, making confocal Raman unique in this field. Moreover, the resolution allows further investigation of the chemistry of individual cells, besides the image of the sample that can be generated thought mapping. This image allows for full spectral information at each pixel, allowing us to see the Raman signature of components within the cell visually. Thus, the confocal Raman technique is valuable in biochemical research.

Mapping can be performed in two ways: either by scanning the light beam along with the sample or by moving the sample itself using the three-dimensional x-y-z axis scanning stage. The main goal of any analytical technique is to obtain the best results. Thus, in terms of microscopy, the ability to distinguish samples details and features, clearly makes this technique unique. With Raman spectroscopy, it is possible to get a good signal from a single point on the sample, dispersing it to a spectrum, and recording the signal using a spectrometer or a multi-channel detector. Raman spectra are recorded over a range of 4000-10 cm^{-1}, but in organic molecules, the Raman active normal modes occur in the 4000-400 cm^{-1} range. [20] Overtones, combination, and difference bands are rare in the Raman spectrum, making it significantly more straightforward than Infrared (IR) counterparts. Raman spectrophotometers can be non-

dispersive or dispersive. A 435.8 nm line of coiled low-pressure mercury arc lamp was used as a light source until the 1960s [20]. Laser sources became available later and replaced the mercury lamp because the laser source provided a stable and intense beam of monochromatic radiation [20].

With a quality confocal Raman microscopy, it is possible to analyze individual particles in dimensions of 1um. In dispersive instruments, a combination of a notch filter and a quality grating monochromator is used called a bandpass filter to isolate a single laser beam. These monochromators are used to separate relatively weak Raman lines from intense Rayleigh scattering radiation with double or triple grating monochromators, rejection filters, super notch filters, holographic notch, or edger filters and holographic filters. [20] Raman spectroscopy is combined to an optical microscope, which lets both a visual and spectroscopic exploration such as either a single point, mapping or imaging measurements. The laser gives a visual inspection of the sample covering an area of about 1x1x5µm. A significant advantage of Raman spectroscopy is the high resolution that can be obtained compared to FTIR, which is about ten ums. The *Gaussian-09* analytical suite software was used for quantum chemical density functional calculations.

Energy optimization was performed before the calculation of the Raman vibrational frequencies. The Becker three hybrid exchange and the lee-Yang-Parr correlation functional, B3LYP, were used in these analyses.[6] A 6-311++G(d, P) basis set was used for calculating the super-molecular form of the combined analytes. A LanL2DZ basis sat, which considers the pseudopotentials for the metal atoms, was employed for SERS simulations. Next, the *Gaussian-09* Raman output data were parsed using an in-house algorithm developed in C++ and subsequently converted to MATLAB version r2016a. Further conversion of Raman activities

into relative Raman intensities following a previously reported procedure was performed. [6] The value of the laser excitation (i.e., 532nm = 18,796.99 cm^{-1}) was used in this recent conversion. Finally, to assist with the data plotting, all Raman peak intensities were normalized by a factor of f=1e^{-10}, and their shapes were modified by applying a Lorentzian band with a full width at half maximum (FWHM) of 7cm^{-1}.

2.5 Experimental Procedure

The Dopamine (C8H11NO2>99%) and Serotonin (C10H12N2O >99%) was purchased from Sigma Aldrich (Milwaukee, WI, USA) and used without further purification. Neurotransmitters mixtures of 5-HT: DA molar ratios 7:3, 1:1, 2:3, 3:7, 1:4 and 1:9, of 10^{-2} M concentrations of the independent serotonin and dopamine in the milli-Q water (of 18.2 MΩ*cm resistivities at room temperature) were first achieved.[6] The mixtures were shaken intensely for a few minutes for best results, then a drop was placed and labeled accordingly in a clean cover glass slips and vacuum dried to avoid any neurotransmitter oxidation. The resulting dried films of the neurotransmitter mixtures were stored under the vacuum until further characterization. The SERS method was used to detect both DA and 5-HT in the nano-molar concentrations successfully.

The process of synthesizing the silver nanoparticles (Ag NPs) used in the SERS method was produced using the classic Lee-Maisel method in a quantity of 100 ML [21]. A 1.7mL of 1% aqueous solution $AgNO_3$ is mixed with a 100 mL of water in a flask with a reflux condenser then to be boiled for 15 minutes. Next, 2 mL of 1% citrate solution is added to the solution mixture. The biochemical solution was kept reflux and being vigorously stirring for one hour and cooled

to room temperature. The resultant AG NPs colloidal suspension in ultra-pure water that was centrifuge several times to remove the excess of organic and unreached impurities before mixing with the combined mixtures of neurotransmitters. Concentrations of DA and 5-HT at 10^{-7} molar, which were obtained by successively diluting each analyte in ultra-pure water used to obtain the mixtures of 4:1, 1:1, and 1:4 ratios. Next, 90 µL of the synthesized Ag NPs solution was mixed with the 10µL of each locution of different ratios of the mixtures of combined neurotransmitters. Finally, each resulting liquid sample bearing Ag NPs and a mixture of neurotransmitters was sonicated for the 20s, then drop-cast on clean labeled cover glass slips, and vacuum dried, to later be stored under the vacuum until characterization. The Raman measurements were acquired using the *alpha 300RAS WITec* confocal Raman system (WITec GmbH, Ulm, Germany).

The 532 nm excitation of a frequency-doubled neodymium-doped yttrium-aluminum-garnet (Nd: YAG) laser, a 1024 × 127-pixel Peltier cooled CCD camera, and an acquisition. The power of the laser was kept a few mW to avoid sample damage of the Raman measurements of the mixtures of the neurotransmitters at 10^{-2} molar concentrations and was restricted to a much lower power output of about 100 µW for SERS measurements. Multiple time series Raman spectra, each of 200 ms, were recorded in the different locations of the neurotransmitter mixtures and averaged. [6] The *WITec Control* 1.60 software was employed for this high-speed data acquisition along with Background subtraction.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Raman data for neurotransmitters mixtures

The label-free detection of DA and 5-HT is mention in various works. However, only a single study implies the chemical interactions between the neurotransmitters and the formation of a new chemical compound as a result. [22] This is a surprising fact, considering their biological proximity and physiological co-existence with the striatum, as well as their similar chemical structure, and no studies have been done. Their probable chemical interactions are taken into account, and as well as continuing our previous work on their simultaneous *in vitro* detection[10], we first explored a possible qualification of 5-HT and DA in the different ratios mixtures.

The method used to provide quantitative information form the mixtures was Raman spectroscopy. However, for easier and more precise analysis in the measurements, the high concentrations of 10^{-2} molar were first considered for such mixtures. The Raman results gathered, of 5-HT and DA alone, and those of 5HT: DA mixtures with ratios of 7:3, 1:1, 2:3, 3:7, 1:4, and 1:9 is represented in Figure 4. While the 5-HT Raman spectrum (blue) has multitude of characteristics vibrations of 463, 602, 759, 835, 940, 1103, 1134, 1235, 1308, 1356, 1435, and 1550 cm^{-1}, with sharp vibrational lines of 264, 395, 597, 750, 795, 935, 1013, 1117, 1148, 1290, 1450, and 1620 cm^{-1} can be seen in the Raman spectrum for DA (Red).

A decrease in intensities of the 5-HT Raman peaks of the mixtures is seen as expected when the amount of 5-HT decreases. On the contrary, the intensities of DA are increasing for the

increasing amounts of DA corresponds well with the increase in the amount of analyte, which can also be observed. These increase/decreases are most visible for the more intense vibrations at 835, 940, 1235, 1356, 1435, and 1550 cm^{-1} for 5-HT, and 750, 795, and 1290 cm^{-1} for DA. Other than the close overlapping for the majority of Raman feature, (i.e. around 602 cm^{-1} for 5-HT and 597 cm^{-1} for DA , 759 cm^{-1} for 5-HT and 750 cm^{-1} for DA , 940 cm^{-1} for 5-HT and 935 cm^{-1} for DA, 1103 cm^{-1} for 5-HT and 1117 cm^{-1} for DA, 1134 cm^{-1} for 5-HT and 1148 cm^{-1} for DA, and 1435 cm^{-1} for 5-HT and 1450 cm^{-1} for DA), there are also

nonoverlapping vibrational lines at 835, 1235, 1308, and 1550 cm^{-1} for 5-HT and at 264, 395, 790, and 1290 cm^{-1} for DA[6].

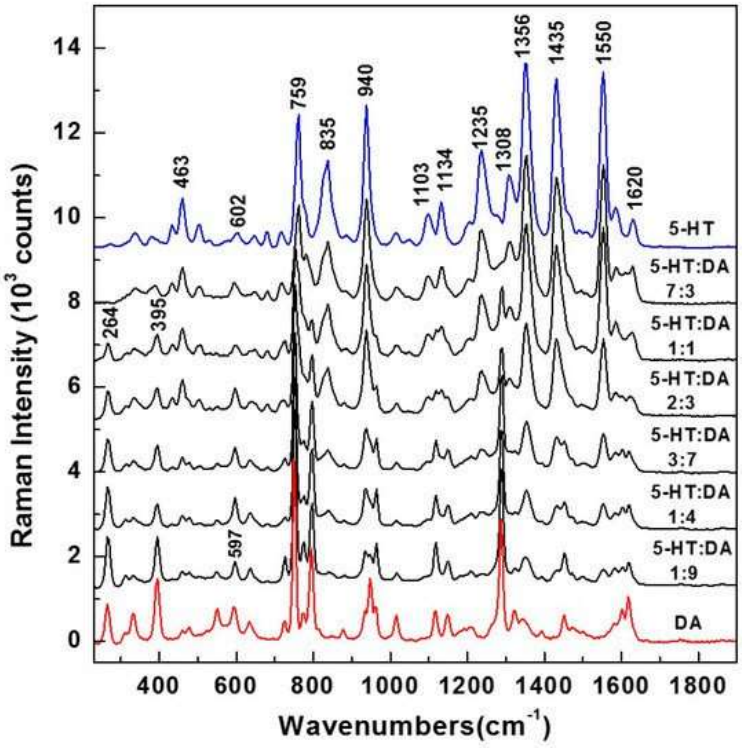

Figure 4. Raman spectra of serotonin (5-HT) and dopamine (DA) at 10^{-2} molar concentrations for their mixtures at different ratios, as labeled. The spectra are vertically translated for easier visualization.[6]

To distinguish between the two analytes, the latter vibrations are used. Not only are the nonoverlapping vibrational lines are used, but all the Raman peaks were considered in the current analysis for better neurotransmitter identification and, more importantly, more accurate quantification presented in Figure 5 with detail. A linear superposition of the Raman spectra of the individual compounds would appear for a mixture of 5-HT and DA in the assumption that the two compounds do not interact chemically.

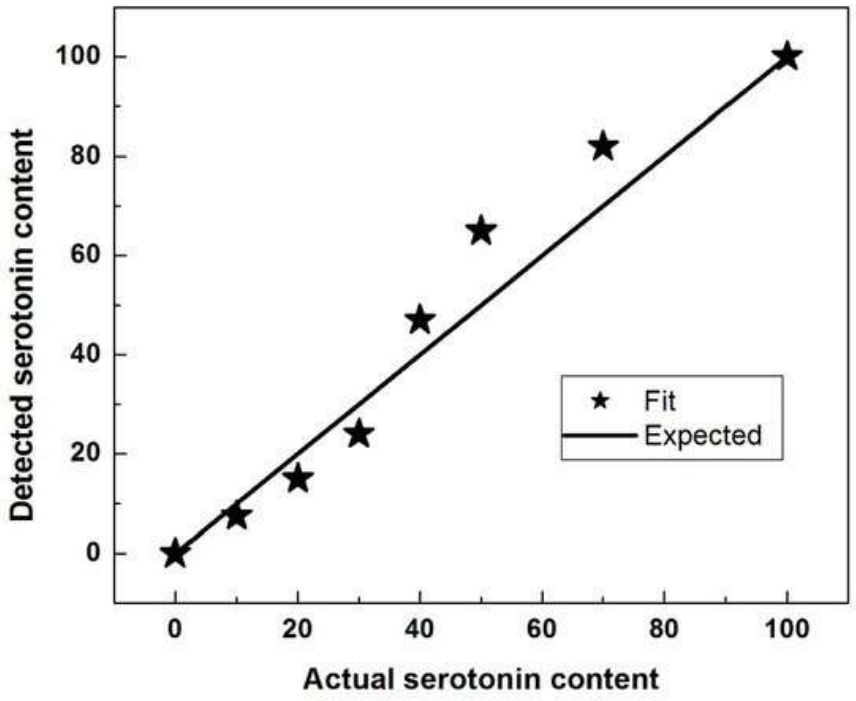

Figure 5. Statistical analysis of the fraction of serotonin detected by fitting Raman measurements versus the actual fraction used in the sample preparation.[6]

However, the data plots indicted by the stars for the sample of Figure 5 were obtained using the prepared be α_o so that the fraction of dopamine in the sample was 1-α_o(by the

horizontal axis scale of Figure 4, the samples have α_o values of 0,0.10, 0.20, 0.30, 0.40, 0.50, 0.70, and 1.0) [6]. Using the variable α, hypothetical linear superpositions of spectra were generated, each consisting of (1-α) times the experimental dopamine spectrum plus α times the experimental serotonin spectrum. Each mixture of 5-HT and DA, a final value of α, was gathered by minimizing the summation of the squared difference between the data collected from the final hypothetical spectrum and the corresponding data of the spectrum that was obtained in the experimental procedure. The stars in the plot in Figure 5 represent the points of the form (α_0, α) where α is a final value from such a fit.

Figure 4 demonstrates a reasonable qualitative estimation of the ratios between serotonin and dopamine that can be obtained accordingly to the difference between the neurotransmitters. The plotted α as a function of α_o, as seen in Figure 5, reveals that, quantitatively, particularly for comparable amounts of neurotransmitters in the mixture determination, is not very accurate. The observation of this inaccuracy suggests a potential formation in the 1:1 ratio compound. This can result in chemical interaction between the 5-HT and the DA molecules. If the quantity δ of this new 1:1 compound formed in the mixture of α_o 5-HT and (1 - α_o) DA, the actual ratio of the mixture should be $\frac{\alpha_o-\delta}{1-\alpha_o-\delta}$, smaller than $\frac{\alpha_o}{1-\alpha_o}$, if $\alpha_o < 0.5$, and larger than $\frac{\alpha_o}{1-\alpha_o}$ if $\alpha_o > 0.5$ [6]. In Figure 5, the fitted value of α, the detected serotonin content, systematically underestimates the serotonin at low 5-HT amounts in the mixtures and overestimates the serotonin at large amounts of mixtures, suggesting the formation of this new compound. A question that arises is how the theoretically calculated Raman spectrum of the 1:1 mixture molecular interaction of the 5-HT

and DA compares to the experimental results shown in Figure 4, and this evaluation is presented in Figure 6.

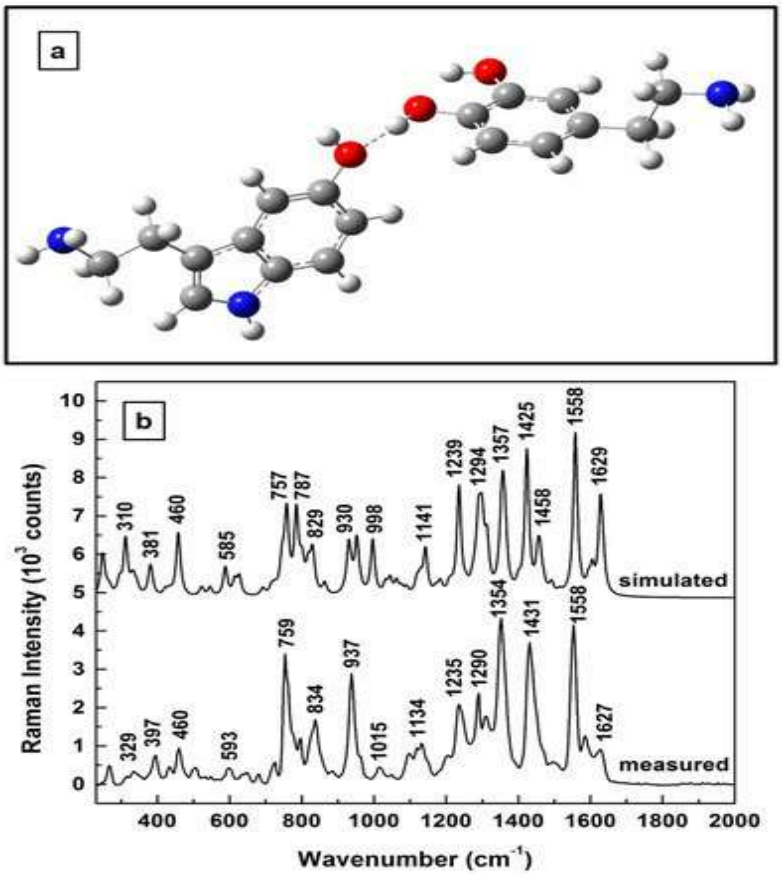

Figure 6 (**a**) A Structural representation of serotonin—dopamine interaction and formation of a new 5-HT—DA complex through hydrogen bonding. Red and blue colors were used for oxygen and nitrogen atoms, respectively. (**b**) Theoretically calculated and experimentally measured Raman vibrations of 5-HT—DA composite. The spectra are vertically translated for easier visualization and appropriately labeled. [6]

As visualized in Figure 6a, the predicted structural representation of this new compound is shown after appropriate energy minimization. A stable interaction happens for stable configurations that occur through hydrogen bonding between the two neurotransmitters. A

serotonin molecule almost perpendicular to the dopamine benzene ring can also be seen in the configuration. Further inspection into the Raman spectra shown in Figure 6b describes the demonstration of good agreement between the computational and experimental determined vibrations with a difference of ±5 cm^{-1} for the position of the most intense vibrational lines. The scaling factor of 0.98 was used for the situated frequencies to overcome the systematic error of ±5 cm^{-1} from the force fields constants employed in the quantum mechanical approaches. Also, vertical translation was done for easier visualization for spectra in order to see the similarities between the two Raman spectra that corroborate the assumption of the formation of a new compound.

3.2 SERS data for 5-HT – DA hydrogen-bonded compound

Measuring the levels of 5-HT and DA using SERS at physiological concentrations, in Figure 7a is presented the energetically optimized molecular structure of the new compound 5-HT – DA hydrogen-bonded complex in the vicinity of the metallic substrate, that is represented by the silver dimer. In Figure 7a, there is a different orientation of the serotonin molecule respect to the dopamine compared to Figure 6a. It can also be observed a sliver dimer planarity with dopamine and quasi-perpendicular respect to serotonin being slightly tilted to the dopamine. Figure 7b again agrees with both theoretical and experimental Raman spectra.

For the measurement, a concentration of 10^{-8} M for the 5-HT-DA composite mixture was used for the analysis in this case, along with a scaling factor of 0.965 was employed to adjust simulated frequencies. There are some variances of ±8cm^{-1} between the positions of some Raman peaks, but most notably, there is a noticeable difference in Figure 7b concerning

the intensities of the features in the 740cm^{-1} – 930 cm^{-1}in comparisons to the 1143 cm^{-1} and 1174 cm^{-1} region.

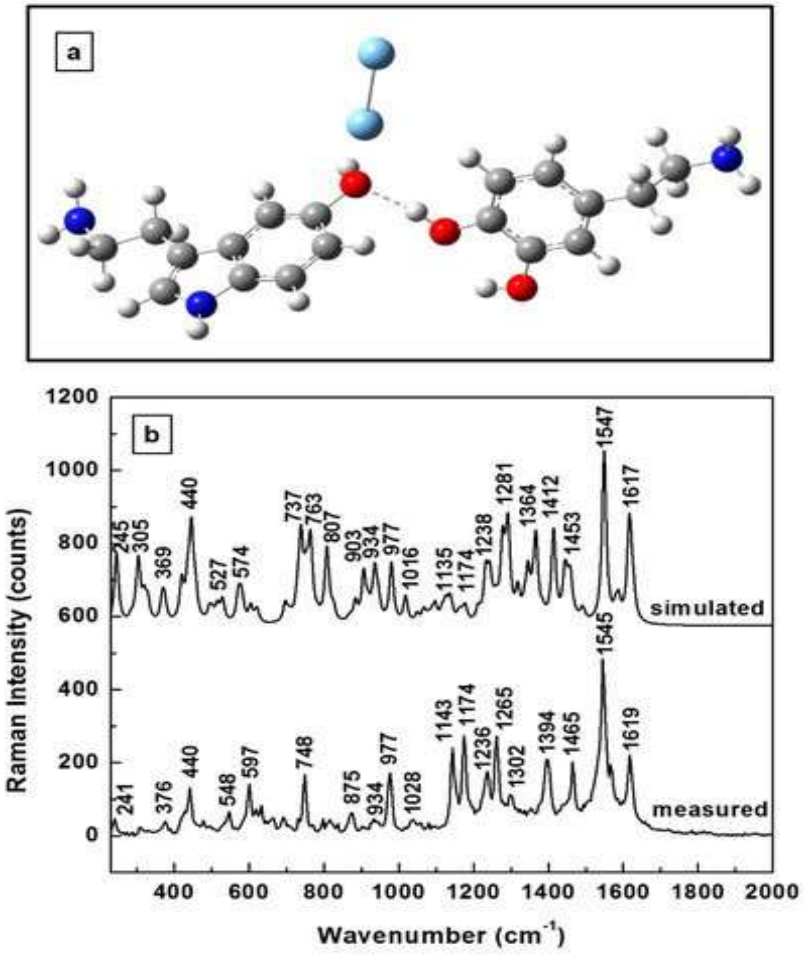

Figure 7. (**a**) Structural representation of new 5-HT—DA composite in the proximity of silver dimer after energy optimization. (**b**) Theoretically estimated and experimentally recorded Raman vibrational spectra of 5-HT—DA composite for a concentration of 10^{-8} M in the proximity of silver.[6]

In the computational spectrum, most of the Raman intensities are higher than those obtained experimentally, but the opposite behavior can be seen in the case of the 1143 and 1174 cm^{-1} vibrations. These Raman peaks belong to the ionic forms of DA and 5-HT, which suggests

an abundance of ionized molecules in the vicinity of the SERS environment. Neurotransmitters are least likely to interact at lower concentrations than at high levels explains the overall lower intensities of the Raman lines experimentally obtained. However, despite the anticipated lower probability of the 5-HT and DA composite formation at weaker interactions, the experimental procedure has an abundance of ionized molecules that needs further analysis in the context of a possible reaction.

The intensity at 748 cm^{-1} in the Raman feature in Figure 7b can be naturally, but not precisely, associated with one of the sharpest vibrational lines of the DA molecule at 750 cm^{-1}. Some vibrational line corresponds to the 5-HT together with the fact that its presence was not detected in the in separate measurements of SERS of low concentrations of dopamine which might belong to the 5-HT-DA bio-composite. This remarks on the importance and necessity of previous investigations of neurotransmitters to accurately identify characteristic vibrational lines in the SERS environment and comprehending its orientation in the proximity of the metallic surface.

Similarly, the perpendicular direction of the silver dimer concerning the 5-HT molecule, a strong vibrational line in the 895cm^{-1} that was previously measured and currently observed in the 895 cm^{-1}. There is a possibility this belongs to the serotonin, but its weakness in the current experimental data might also imply its association with the interaction of the neurotransmitters with each other with the new composite formation, but its source certainly forms the serotonin in the 5-HT-DA bio complex. Obtained vibrations, such as 440 and 977 cm^{-1}, that were previously reported for a planar orientation of the silver dimer respect to that of dopamine quinone molecule (observed in the 440 and 958 cm^{-1}), suggestion association with the features of dopamine quinone of the DA.

The sharp vibrational lines of the currently observed 1547 cm^{-1}Raman peak are like 1527 cm^{-1} line of the DA anion [6], again for its similar planar orientation in the proximity with the silver dimer, this ionic DA form is the main contributor to this line. Although all these vibrational are associated with their neurotransmitters, the ±20 cm^{-1}large shifts still imply a new compound formation. We present Figure 8 to show overall averages of the 140 SERS spectra for better comprehension of the probable creation of a 5-HT-DA compound and discrimination of these low concentrations of about 10^{-9} M.

The data were mainly obtained from the 4:1, 1:1, and 1:4 for the 5-HT: DA ratios in different locations of the sample. For an easier comparison of the SERS average mixture spectrum with those of the 5-HT and DA, it can be seen in Figure 8 the SERS spectra of each of the neurotransmitters; the blue line belonging to serotonin and the red line to dopamine.

In the spectra, it is revealed that concentrations close to physiological levels, even in the spectra of the standard neurotransmitter is based on vibrations at 482, 675, and 1057 cm^{-1} for 5-HT, and the strong vibrations at 1174 cm^{-1} for DA expected when the neurotransmitters when dissolved in water in the sample preparation. There is also a notably decreasing trend in intensity of the 1174 cm^{-1} lines with the 5-HT addition. This observation also implies that serotonin has an antioxidant effect on dopamine besides the probability of the two neurotransmitters forming a new compound. Deprotonation of DA and transformation to dopamine quinone is compensation by the addition of serotonin and the hydrogen sharing form the two analytes. There is also evidence of the 5-HT-DA compound formation in the weak SERS Raman lines 440, 752 and 984 cm^{-1}.

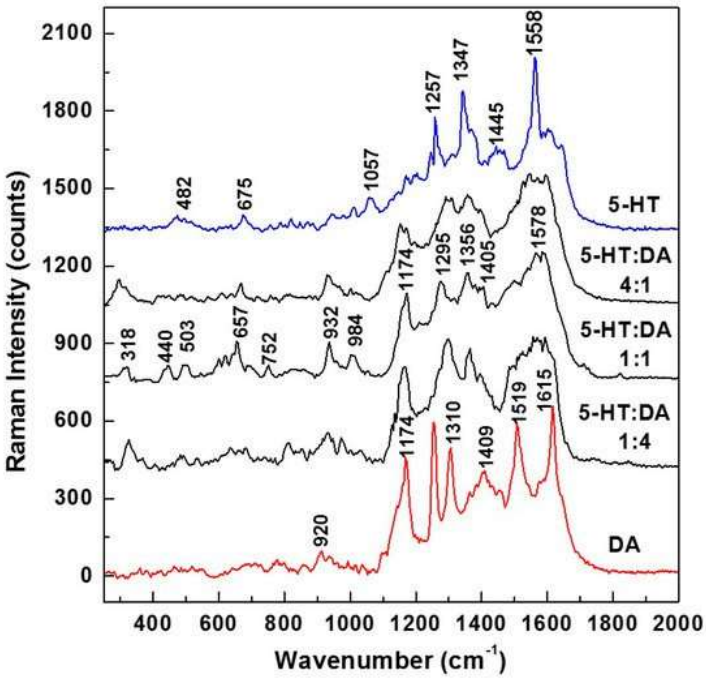

Figure 8. Overall, averages of 140 Raman spectra recorded in different spots on SERS mixture samples with different ratios, as labeled (seven various time series acquisitions, of 20 spectra each and at 200 ms per spectrum). The individual Raman spectra of 5-HT and DA are also presented for comparison.[6]

3.3 Conclusion

The brain contains about 100 billion neurons communicating through relative long distances to other cells. Neurons are composed of fibers called dendrites and an axon that is responsible for sending signals in the form of an action potential. [1] Neurotransmitters are molecules used in the nervous system to transmit information between neurons.

Two of the most important neurotransmitters that are present in the central nervous system are Dopamine (DA) and Serotonin (5-hydroxytryptamine or 5-HT). DA functioning as a motivational component for reward-motivated behavior and 5-HT helps regulate mood and behavior. [7] The two modulators are primarily centered in the midbrain: DA having the VTA

and the nigrostriatal as its primary pathways and the 5-HT having the dorsal and medial raphe nuclei as its central pathway. Thus, from a biological perspective, their interaction is expected because the DRN contains striatal and cortex regions projected with the median raphe nuclei entering the limbic area, revealing the proximity of the neurotransmitters. Furthermore, DA and 5-HT have hydroxyl groups that are suitable for intermolecular interactions among themselves. However, experimental data through optical methods have not yet been reported for the analyte interaction, primarily because they have been tagged with a variety of fluorescent dyes nanoparticles, making such measurements impossible.

At relatively high concentrations of DA and 5-HT, Raman Spectroscopy is known to be a sensitive method for label-free detection of these analytes at molecular levels [10]. However, if measurements at physiological levels (low concentrations) are desired, Surface-Enhanced Raman Spectroscopy (SERS) needs to be employed. A plasmonic silver material was used in the current work for the amplification of the electric field and enhancement of Raman signal. In this context is worth mentioning that one challenging task for SERS label-free detection is that at the metallic SERS surfaces chemical interactions arise, that changing the frequency and intensity of vibrational lines. Quantum chemical density functional calculations were obtained using *Gaussian-09* analytical suite software. The Raman measurements were acquired for the *alpha 300RAS WITec* confocal Raman system.

Although label-free detection of DA and 5-HT is mention in various works, only a single study implies the chemical interactions between the neurotransmitters and the formation of a new chemical compound. [22] Thus, in the current work, the probable chemical interactions are first considered, at a high concentration of the analytes. We also explore a possible qualification of DA and 5-HT in different ratio mixtures of 7:3, 1:1, 2:3, 3:7, 1:4 and 1:9. As expected, the

results show a decrease in intensities of the 5-HT Raman peaks when the 5-HT amount is decreased. On the contrary, the DA intensities are increasing for the increasing amounts for DA, also as expected. In order to distinguish between the two analytes, characteristics vibrations were used for each. A quantitative statistical data plot presented in the current work reveals that, at comparable amounts of the analytes in the mixture (ratio 1:1) potential formation of a composite might arise from the interaction between 5-HT and DA. Later the theoretical calculated Raman spectrum of the 1:1 and comparison with the experimental result proves this assumption. This interaction happens through hydrogen bonding between the two neurotransmitters. Since only through SERS we can obtain detection of neurotransmitters at physiological levels, we also engaged in this more demanding investigation, again by a theoretical and experimental comparison approach.

The overall averages of 140 SERS spectra recorded in different locations in the samples for 5-HT-DA of 4:1, 1:1 and 1:4 ratio mixtures were analyzed and discussed. The Raman spectra reveal that at concentrations close to physiological levels vibrations associated with their ionic forms are seen at 482, 675, and 1057 cm^{-1} for 5-HT, and the strong vibrations at 1174 cm^{-1} for DA. It is also implied an antioxidant effect of serotonin on dopamine in the decreasing trend in intensity of 1174 cm^{-1} lines with the 5-HT addition. However, qualifications of the two analytes cannot yet be accurately done.

In conclusion, the finding of the probable formation of the 5-HT-DA compound can open new avenues for pharmaceutical and drug research. The interaction of DA and 5-HT is indeed confirmed in this study at high millimolar neurotransmitter concentrations, but less accurate at physiological levels. More research is required to fully determine the chemical structure and biological applications of this new compound.

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CURRICULUM VITA

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