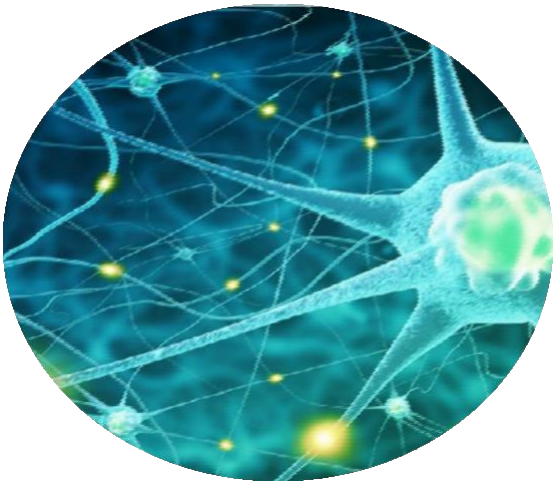
AGRICULTURAL UNIVERSITY OF ATHENS
FACULTY OF APPLIED BIOLOGY AND BIOTECHNOLOGY

DEPARTMENT OF BIOTECHNOLOGY
LABORATORY OF CELL TECHNOLOGY

PhD Thesis

Development of advanced cellular and molecular biosensors for the study of neurotransmitter interaction and prospects for applications in Biology and Medicine



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‘’Ανάπτυξη προηγμένων κυτταρικών και μοριακών βιοαισθητήρων για τη μελέτη αλληλεπίδρασης νευρομεταβιβαστών και προοπτικές εφαρμογών στη Βιολογία και την Ιατρική’’

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Development of advanced cellular and molecular biosensors for the study of neurotransmitter interaction and prospects for applications in Biology and Medicine

Department of Biotechnology
Laboratory of Cell Technology

Abstract

The purpose of this thesis is to study the effect of neurotransmitters on nerve cells and to develop advanced methods in the field of Neurobiology.

Neurotransmitters are a group of chemicals released by nerve endings that allow transmission of cell-to-cell signals. They can also be named as a chemical messenger or chemical transmitter because of their ability to transmit nerve impulses from one cell to the next throughout the nervous system. They play an important role in shaping everyday life and functions. Their precise number is unknown, but more than 200 chemical messengers have been uniquely recognized.

In recent years, a system of measuring the change in electrical potential of a single cell membrane or single-cell segment but at the cell population/tissue level has been developed. The Bioelectric Recognition Assay (BERA) is a method for detecting viruses and other bioactive substances based on the measurement of changes in the electrical properties of a cell group properly. It is possible to detect human and plant viruses in a specific, fast (less than 3 minutes), remarkably reproducible and economical way. The sensitivity of BERA is comparable to previous immunological, cytological and molecular techniques.

This doctoral thesis is divided into two sections. The first section deals with the investigation of the effect of dopamine on nerve cells and more specifically the development of a functional test for the study of the in vitro interaction of dopamine neurotransmitters with dopamine receptor-bearing nerve cells for the purpose of evaluating different drug targets. The idea is based on the extremely rapid measurement of changes observed in the electrical properties of cultured mouse neuroblastoma (N2a) cells. Analysing the experimental data, a relatively close relationship was observed between dopamine concentration and cell membrane potential. At lower concentrations of dopamine (nanomolar) membrane depolarization was observed, while at higher concentrations (micromolar) cell membrane hyperpolarization was observed. The use of eticlopride, a dopamine D2-receptor antagonist, resulted in concentration-dependent membrane depolarization. In addition, a significant attenuation of cell membrane

hyperpolarization was observed when cells were treated with sodium chloride. As determined by cyclic voltammetry, the bioelectric response to dopamine was highly correlated with the pattern of dopamine overflow by N2a cells. The novel approach was additionally used to evaluate the dopaminergic activity of chaste tree extracts (Vitex agnus-castus) as well as the activity of two well-known antipsychotics, haloperidol and olanzapine. This new concept offers many promising prospects for the development of an advanced system for the rapid functional characterization of neurotransmitters agonists and antagonists.

The second section deals with the examination of the existence of non-chemical, distant cellular interactions between neuronal cells. The mechanism of cell-to-cell communication and the coordinated cellular responses they present are something that has been of great concern to the scientific community in recent years. The work in the present study focuses on non-chemical, distant cellular interactions (NCDCI) that are likely to be responsible for this communication. Recent experiments have suggested the field theory of conscious electromagnetic information (CEMI) as a possible explanation for this cell-to-cell communication. In the present work, using a bioelectric biosensor, we observed the emergence of distant communication between neuroblastoma cells providing evidence for this theory. This observation was made simultaneously with the observations of changes in the membrane potential of human SK-N-SH neuroblastoma cells that were physically separated from each other. The cells were divided into two groups naturally separated. In one group we had the "inducer" cells that were stimulated with dopamine, and in the other group, we had the "detector" cells which showed a synchronized response to the "inducer" cells, with the amplitude of the response decreasing as the distance increased. In order to investigate the nature of the mechanisms that cause the observed distant cell interactions, cell cultures were separated with barriers, which were non-transparent in certain frequency ranges of the electromagnetic radiation spectrum or treated with vinblastine, a vinca alkaloid, which binds tubulin, thereby inhibiting the assembly of microtubules. The mechanism responsible for cell-to-cell communication is discussed in accordance with the observed effects of coordinated changes in membrane potential.

Scientific area: Neurotransmitters

Keywords: Bioelectric Recognition Assay (BERA), biosensor, dopamine

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Τμήμα Βιοτεχνολογίας
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Περίληψη

Ο σκοπός της παρούσας διδακτορικής διατριβής είναι η μελέτη της επίδρασης των νευροδιαβιβαστών σε νευρικά κύτταρα και η ανάπτυξη προηγμένων μεθόδων στον τομέα της Νευροβιολογίας.

Οι νευροδιαβιβαστές είναι μια ομάδα χημικών ουσιών που απελευθερώνονται από τις νευρικές απολήξεις και επιτρέπουν τη νευροδιαβίβαση, δηλαδή την ανταλλαγή σημάτων μεταξύ των νευρικών κυττάρων. Επίσης ονομάζονται ως χημικός αγγελιοφόρος ή χημικός πομπός λόγω της ικανότητάς τους να μεταδίδουν τα νευρικά σήματα από ένα νευρικό κύτταρο σε όλο το νευρικό σύστημα. Ο ακριβής αριθμός τους είναι άγνωστος, αλλά περισσότεροι από 200 χημικοί αγγελιοφόροι έχουν αναγνωριστεί.

Τα τελευταία χρόνια αναπτύχθηκε ένα σύστημα μέτρησης της μεταβολής του ηλεκτρικού δυναμικού μιας κυτταρικής μεμβράνης. Η Βιοηλεκτρική Μέθοδος Αναγνώρισης (Bioelectric Recognition Assay - BERA) είναι μια μέθοδος για την ανίχνευση ιών και άλλων βιοδραστικών ουσιών με βάση τη μέτρηση των αλλαγών στις ηλεκτρικές ιδιότητες μιας κυτταρικής ομάδας. Είναι δυνατό να ανιχνευθούν ανθρώπινοι και φυτικοί ιοί με συγκεκριμένο, γρήγορο (λιγότερο από 3 λεπτά) τρόπο, αξιοσημείωτα αναπαραγώγιμο και οικονομικό. Η ευαισθησία της μεθόδου BERA είναι συγκρίσιμη με προηγούμενες ανοσολογικές, κυτταρολογικές και μοριακές τεχνικές.

Η παρούσα διδακτορική διατριβή χωρίστηκε σε δυο ενότητες. Η πρώτη ενότητα αφορά την διερεύνηση της επίδρασης της ντοπαμίνης σε νευρικά κύτταρα και πιο συγκεκριμένα την ανάπτυξη μιας λειτουργικής δοκιμής για τη μελέτη της in vitro αλληλεπίδραση του νευροδιαβιβαστή ντοπαμίνη με νευρικά κύτταρα που φέρουν υποδοχείς ντοπαμίνης, με σκοπό την αξιολόγηση διαφόρων φαρμάκων που έχουν σαν στόχους υποδοχείς ντοπαμίνης. Η ιδέα βασίζεται στην εξαιρετικά ταχεία μέτρηση των αλλαγών που παρατηρούνται στις ηλεκτρικές ιδιότητες των καλλιεργημένων κυττάρων νευροβλαστώματος ποντικού (N2a). Αναλύοντας τα πειραματικά δεδομένα, παρατηρήθηκε μια σχετικά στενή σχέση μεταξύ της συγκέντρωσης ντοπαμίνης και του δυναμικού κυτταρικής μεμβράνης. Σε χαμηλές συγκεντρώσεις παρατηρήθηκε αποπόλωση της κυτταρικής μεμβράνης, ενώ σε υψηλότερες συγκεντρώσεις

παρατηρήθηκε υπερπόλωση κυτταρικής μεμβράνης. Η χρήση της ετικλοπρίδης, ενός ανταγωνιστή των D2-υποδοχέων της ντοπαμίνης, οδήγησε σε εξαρτώμενη από τη συγκέντρωση αποπόλωση της μεμβράνης. Επιπλέον, παρατηρήθηκε σημαντική εξασθένιση του φαινομένου της υπερπόλωσης της κυτταρικής μεμβράνης όταν τα κύτταρα υποβλήθηκαν σε αγωγή με χλωριούχο νάτριο. Όπως προσδιορίζεται από την κυκλική βολταμετρία, η βιοηλεκτρική απόκριση στην ντοπαμίνη συσχετίζεται εξαιρετικά με το πρότυπο "υπερχείλισης" της ντοπαμίνης από τα κύτταρα N2a. Η νέα προσέγγιση χρησιμοποιήθηκε επιπρόσθετα για την αξιολόγηση της ντοπαμινεργικής δραστικότητας των εκχυλισμάτων του φυτού λυγαριά (*Vitex agnus-castus*) καθώς και της δραστικότητας δύο γνωστών αντιψυχωτικών, της αλοπεριδόλης και της ολανζαπίνης. Αυτή η νέα ιδέα προσφέρει πολλές ελπιδοφόρες προοπτικές για την ανάπτυξη ενός προηγμένου συστήματος για τον γρήγορο χαρακτηρισμό των αγωνιστών και ανταγωνιστών των νευροδιαβιβαστών,

Η δεύτερη ενότητα αφορά τον έλεγχο της ύπαρξης μη χημικών, μακρινών κυτταρικών αλληλεπιδράσεων μεταξύ νευρικών κυττάρων. Ο μηχανισμός της επικοινωνίας μεταξύ κυττάρου προς κύτταρο και οι συντονισμένες κυτταρικές αποκρίσεις που παρουσιάζουν είναι κάτι που έχει απασχολήσει ιδιαίτερα την επιστημονική κοινότητα τα τελευταία χρόνια. Οι περισσότερες εργασίες επικεντρώνονται στις μη χημικές, μακρινές κυτταρικές αλληλεπιδράσεις (NCDCI) που είναι πιθανό να είναι υπεύθυνες για αυτήν την επικοινωνία. Πρόσφατα πειράματα έχουν προτείνει την θεωρία πεδίου της συνειδητής ηλεκτρομαγνητικής πληροφορίας (CEMI) ως πιθανή εξήγηση για αυτή την επικοινωνία κυττάρου προς κύτταρο. Στην παρούσα εργασία, χρησιμοποιώντας έναν βιοηλεκτρικό βιοαισθητήρα, παρατηρήσαμε την εμφάνιση μακρινής επικοινωνίας μεταξύ κυττάρων νευροβλαστώματος τα οποία παρέχουν στοιχεία για αυτή τη θεωρία. Αυτή η παρατήρηση έγινε ταυτόχρονα με τις παρατηρήσεις μεταβολών στο δυναμικό μεμβράνης των ανθρώπινων κυττάρων νευροβλαστώματος SK-N-SH που φυσικά διαχωρίστηκαν μεταξύ τους. Τα κύτταρα χωρίστηκαν φυσικά σε δύο ομάδες. Σε μία ομάδα είχαμε τα "επαγωγικά" κύτταρα που διεγέρθηκαν με ντοπαμίνη, και στην άλλη ομάδα, είχαμε τα κύτταρα "ανιχνευτή" που έδειξαν συγχρονισμένη απόκριση στα κύτταρα "επαγωγέα", με το εύρος της απόκρισης να μειώνεται όταν η απόσταση αυξανόταν. Για να διερευνηθεί η φύση των μηχανισμών που προκαλούν τις παρατηρούμενες αλληλεπιδράσεις των κυττάρων, οι κυτταρικές καλλιέργειες διαχωρίστηκαν με φραγμούς που επιτρέπουν διαφορετικά μήκη κύματος να τους διαπερνούν ή υπεβλήθησαν σε επεξεργασία με βινβλαστίνη, ένα αλκαλοειδές, το οποίο δεσμεύει τη τουμπουλίνη, αναστέλλοντας έτσι τη συναρμολόγηση

των μικροσωληνίσκων. Ο μηχανισμός που είναι υπεύθυνος για την επικοινωνία κυττάρου προς κύτταρο συζητείται σύμφωνα με τις παρατηρούμενες επιδράσεις των συντονισμένων αλλαγών στο δυναμικό της μεμβράνης.

Επιστημονική περιοχή: Νευροδιαβιβαστές

Λέξεις-κλειδιά: Δοκιμασία Βιοηλεκτρικής Αναγνώρισης (BERA), βιοαισθητήρας, ντοπαμίνη

Development of advanced cellular and molecular biosensors for the study of neurotransmitter interaction and prospects for applications in Biology and Medicine

Η αποδοχή για εκπόνηση της παρούσας Διδακτορικής Διατριβής έγινε μετά από απόφαση της Γενικής Συνέλευσης του Τμήματος Βιοτεχνολογίας του Γεωπονικού Πανεπιστημίου Αθηνών (ΓΣΕΣ, 9η/11-4-2014). Ο ορισμός της τριμελούς επιτροπής καθώς και η ανάθεση του θέματος έγιναν μετά από αποφάσεις των ΓΣΕΣ (10η/16-5-2014) και (11η/13-6-2014), αντίστοιχα. Η επταμελής εξεταστική επιτροπή ορίστηκε κατά την (7η/16-12-2020) ΓΣΕΣ

PhD Thesis

Development of advanced cellular and molecular biosensors for the study of neurotransmitter interaction and prospects for applications in Biology and Medicine

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List of publications

Publications leading to this thesis

Apostolou T., Moschopoulou G., Kolotourou E., Kintzios S. (2017) Assessment of in vitro dopamine-neuroblastoma cell interactions with a bioelectric biosensor: perspective for a novel in vitro functional assay for dopamine agonist/antagonist activity. Talanta 170:69-73

Apostolou T., Kintzios S. (2018) Evidence of near-instantaneous distant, non-chemical communication between neuronal (human SK-N-SH neuroblastoma) cells by using a novel bioelectric biosensor. Journal of Consciousness Studies 25: 62-74(13)

Other publications and conference announcements during the course of this PhD

Paivana G., **Apostolou T.**, Kaltsas G., Kintzios S. (2017) Study of the dopamine effect into cell solutions by impedance analysis. Journal of Physics: Conference Series 931(1),012010

Paivana G., **Apostolou T.**, Mavrikou S., Barmpakos D., Kaltsas G., Kintzios S. (2019) Impedance study of dopamine effects after application on 2D and 3D neuroblastoma cell cultures developed on a 3D printed well. Chemonsensors 7:6

Apostolou T, Mavrikou S, Denaxa N.-K, Paivana G, Roussos P.A, Kintzios S, (2019) Assessment of Cypermethrin Residues in Tobacco by a Bioelectric Recognition Assay (BERA) Neuroblastoma Cell-Based Biosensor. Chemosensors 7:58

Kountanis T., Paivana G., **Apostolou T**., Kintzios S., Kaltsas G. Impedance Analysis for Cell Discrimination and Pesticides' Concentration Determination, International Conference 'Science in Technology' SCinTE 2015.

Αποστόλου Θ. Ανάπτυξη προηγμένων κυτταρικών και μοριακών βιοαισθητήρων για τη μελέτη αλληλεπίδρασης νευρομεταβιβαστών και προοπτικές εφαρμογών στην Βιολογία και την Ιατρική, 1η Ημερίδα Παρουσίασης της έρευνας Υπ. Διδακτόρων της Σχολής Τροφίμων, Βιοτεχνολογίας και Ανάπτυξης, 8 Δεκεμβρίου 2016, Αθήνα.

Paivana G., **Apostolou T.**, Kaltsas G., Kintzios S. Study of the dopamine effect into cell solutions by impedance analysis, Conference on Bio-Medical Instrumentation and related Engineering and Physical Sciences, BIOMEP 2017, Athens.

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Finally, I would like to wholeheartedly thank my family for their unequivocal support at all levels. I hope the result justifies their own hopes. This thesis is dedicated to the memory of my father, who did not see it in its final form.

Στην μνήμη του πατέρα μου

08 Φεβρουαρίου 2020

Table of Contents

Chapter 1

General Introduction

1.1 Neurotransmitters

Neurotransmitters are a group of chemical substances released by nerve endings that allow neurotransmission. They can be also named as a chemical messenger or chemical transmitter because of their ability to transmit nerve impulses from one cell (nerve cell) to the next throughout the nervous system (Lodish et al., 2000). Neurotransmitters are synthesized by neurons and released through the presynaptic membrane from synaptic vesicles into the synaptic cleft, where they then bind to neurotransmitter receptors on the target cells. Various neurotransmitters are synthesized from abundant precursors such as amino acids, which are easily obtainable from the nutrition and only need a short number of biosynthetic steps for conversion. They play an important role in shaping everyday life and functions. Their precise amounts are unknown, but more than 200 chemical messengers have been uniquely recognized (Verkhratsky et al., 2009).

Discovery

Till the beginning of the 20th century, was believed that brain neurons communicate via the synapse with electrical signals. Nevertheless, in 1921 the German pharmacologist Otto Loewi (1873–1961) proved that neurons can communicate by releasing chemicals. In his experiment, he used two frog hearts. He isolated the frog hearts by holding the nerves in only one heart. Then he placed the hearts in two different chambers, connected them, and filled them with Ringer's solution. First, he caused a stimulation of the vagus nerve for a short time and then he transferred the Ringer's solution, which was in the chamber with the first heart at the time of stimulation, to the chamber with the second heart. He then noticed that heartbeats decreased as if the vagus nerve had been stimulated. He repeated the above experiment, but this time the accelerator nerve was stimulated, and by transferring the solution to the second heart he noticed that the beats increased (Loewi, 1960). Summarizing the experimental data, Loewi hypothesized that a chemical was released into the solution of the first chamber, which was transferred to the second chamber, after the electrical stimulation of the vagus nerve. He called this chemical "Vagusstoff", which nowadays we know as the neurotransmitter called acetylcholine (Saladin, 2009).

Mechanism

Neurotransmitters are synthesized by the ribosomes of the pre-synaptic neurons, stored in synaptic vesicles and gathered close to the cell membrane at the axon terminal of the presynaptic neuron. The calcium channels that are located in the cell membrane of the presynaptic neurons are opening after the stimulation of the presynaptic neuron. This opening causes calcium ions to enter into the cell and provoke the synaptic vesicles to merge with the pre-synaptic membrane, and consequently releases the neurotransmitters into the synaptic cleft which is the space between two neurons (Elias et al., 2005).

A released neurotransmitter is typically available in the synaptic cleft for a short time before it is metabolized by enzymes, reuptaken by the presynaptic neuron, or attached to a postsynaptic receptor. A lot of neurotransmitters' receptors are found in the post-synaptic cell membrane of the receiving neuron. Neurotransmitters bind on these receptors, which basically are large protein molecules. These receptors and neurotransmitters are acting like locks and keys. Only some specific keys can match with certain locks. Notwithstanding, short-period exposure of the receptor to a neurotransmitter is enough for generating a postsynaptic response by way of synaptic transmission.

Binding of neurotransmitters can affect the function of a neuron in two ways: an excitatory or inhibitory. Some examples of neurotransmitters that generally act as excitatory neurotransmitters are glutamate and acetylcholine. These neurotransmitters have the ability to bind on the post-synaptic neurons' receptors and due to this binding, the sodium channels are opening. The opening of the sodium channels causes the moving of sodium ions from outside of the cell into the cell because of the higher concentration of sodium ions outside the cell. Finally, this sodium ions' movement produces an action potential, which generates a nerve impulse. On the other hand, the inhibitory neurotransmitters (e.g. dopamine, GABA), like the excitatory neurotransmitters, bind on the post-synaptic neurons' receptors with results in the opening of potassium channels or chloride channels. When potassium channels are opening, potassium ions move out of the cell and hyperpolarization occurs, and when chloride channels are opening, a significant amount of chloride ions is moving inside the cell (Figure 1). By opening these channels the generation of the action potential is inhibited (Whishaw et al., 2014)

Identification

Over the years neuroscientists set up a few guidelines or criteria that should be met from a substance in order to be named as a neurotransmitter:

- The neurotransmitter must be released in a pharmacologically or chemically active form, from nerve terminals after stimulation, and should be possible to isolate it and describe its structure.
- The substance must be synthesized and released from neurons. Hence, the presynaptic neuron must include both the transmitter and the proper enzymes required to synthesize that substance.
- After the release of the substance, an active mechanism should exist in order to terminate the action of the neurotransmitter. Such mechanisms are the enzymatic inactivation of the neurotransmitter or the uptake of the transmitter by the presynaptic neurons.
- When a neurotransmitter is applied on the postsynaptic cell, it should reproduce the same effects that are seen when it is released by a neuron.

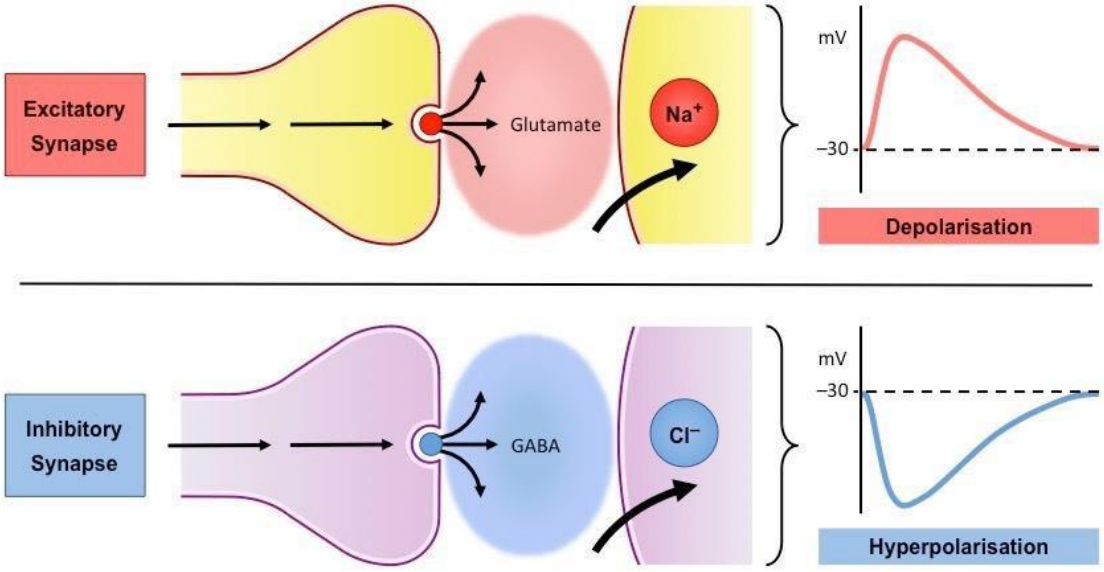


Figure 1. *Schematic representation of the mode of action of excitatory and inhibitory neurotransmitters (Figure adapted from http://ib.bioninja.com.au/Media/epsp-vs-ipsp_medjpeg).*

Types

Neurotransmitters can be classified in many and different ways. Classifying them into monoamines, peptides and amino acids is sufficient for some purposes. The most

well-known neurotransmitters are shown in Table 1.

Table 1. *The major known neurotransmitters and their location*

Neurotransmitter(s)	Precursor Molecules (s)	Predominant Postsynaptic Effect	Location (s)
Acetylcholine	Acetyl-CoA and choline	Excitatory	CNS*: brain and spinal cord PNS**:neuromuscular junction and ANS
Biogenic Amines			
Catecholamines (norepinephrine, epinephrine, dopamine)	Tyrosine	Excitatory	CNS: brain and spinal cord PNS: ANS (sympathetic division)
Serotonin	Tryptophan	Excitatory	CNS: brain
Histamine	Histidine	Excitatory	CNS: brain
Amino Acids			
Glutamate	Glutamine	Excitatory	CNS: brain (major neurotransmitter of the brain)
GABA (γ-aminobutyric acid)	Glutamate	Inhibitory	CNS: brain and spinal cord
Glycine	Serine	Inhibitory	CNS: brain and spinal cord (most common inhibitory neurotransmitter in the spinal cord)
Neuropeptide Y	---	Excitatory & Inhibitory	CNS: brain PNS: ANS

*CNS: Central nervous system, **PNS: Peripheral nervous system

As mentioned before, a neurotransmitter is able to bind in a receptor and cause the activation of it. Hence, the results of this activation hinge on the chemical properties of the receptors and the connections of the neurons that utilize the transmitter. In Table 2 some examples of important neurotransmitter actions are presented:

Table 2. *Functions of most important neurotransmitters*

Neurotransmitters	Primary Function	Receptors	Notes
Glutamine	A major excitatory neurotransmitter; involved in memory.	NMDA, others	Oversupply can overstimulate the brain, producing migraines or seizures (which is why some people avoid monosodium glutamate in food)
GABA (γ-aminobutyric acid)	A major inhibitory neurotransmitter.	GABAA, GABAB	Mediates muscle tone, Receptors susceptible to alcohol which creates CNS depression. Undersupply linked to seizures, tremors and insomnia.
Acetylcholine	Muscle control, memory formation, sensory response, excitatory neurotransmitter	Nicotinic, muscarinic	One of the most common, very well studied. A major player in memory. Imbalance cause twitching or paralysis.
Dopamine	Influences movement, learning, attention and emotion	D1, D2, D3, D4, D5	Oversupply linked to schizophrenia. Undersupply linked to tremors and decreased mobility in Parkinson's disease. Cocaine and opiates have a significant effect on its release.
Serotonin	Intestinal movement control, mood regulation, appetite, sleep, muscle control	5-HT	Undersupply linked to depression. Most antidepressants mimic the effect of serotonin. Most narcotics affect its release or reuptake.
Norepinephrine	Fight or Flight response	Adrenergic	Produced from Dopamine in the adrenal glands (on kidneys). Undersupply can depress mood.

1.1.1 Dopamine

Structure

A dopamine molecule consists of catechol in which the hydrogen at position 4 is substituted by a 2-aminoethyl group. Dopamine belongs to the catecholamine family together with the neurotransmitters norepinephrine and epinephrine. The attachment of an amine in the benzene ring that is present in the structure, makes dopamine a substituted phenethylamine, which is a family that includes numerous psychoactive drugs (Cartel et al., 1982).

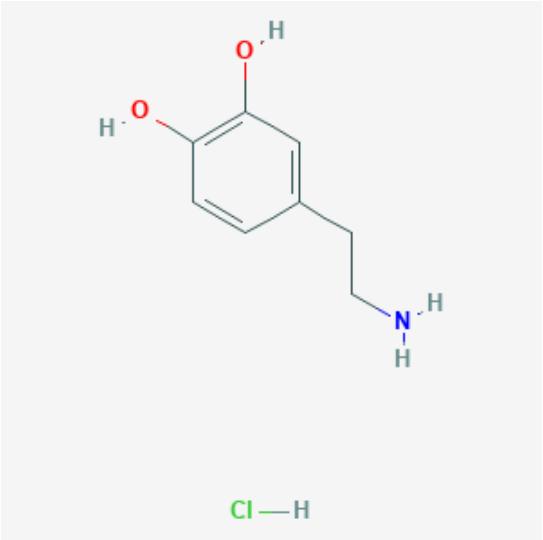


Figure 2. *Schematic representation of dopamine molecule' chemical structure. (Figure adapted from https://pubchem.ncbi.nlm.nih.gov/compound/Dopamine#section=2D-Structure)*

Dopamine is an organic base which, like all organic bases, protonates when found in acidic environments. The main feature of this form is that while it is fairly stable and relatively soluble in water, it tends to oxidize relatively easily when exposed to oxygen or other oxidants. Unlike acidic environments, when found in basic environments, it is not protonated resulting in it being more reactive and therefore less soluble in water. Dopamine usual is provided for use in pharmaceuticals or as a chemical in the form of dopamine hydrochloride, as its protonated form is readily water-soluble and fairly stable. Dopamine hydrochloride is provided in a dry form and is a relatively colorless powder (Cartel et al., 1982).

Dopamine is one of the major monoamine neurotransmitters in the mammalian brain. It acts as a neurotransmitter but also and as hormone and plays an important role in both body and brain functions. Dopamine is generated by hydroxylation and decarboxylation of the amino acid tyrosine and then metabolized into epinephrine and norepinephrine.

In the brain, dopamine functions as a neurotransmitter. The role that dopamine plays in brain function was first put forward by the Nobel laureate, Arvid Carlsson. Later, Oleh Hornykeiwicz and his colleagues showed for the first time that in the brain of patients with Parkinson's there was a lack of dopamine. Dopamine has been shown to control numerous functions likes reward, emotion, movement, and cognition. The anticipation of

most types of rewards increases the level of dopamine in the brain (Berridge et al., 2009) and several addictive drugs increase dopamine release or block its reuptake into neurons following release.

In addition to its effects on the nervous system, dopamine has the potential to act as a chemical messenger between different peripheral systems:

- For example, it has the ability to suspend the release of norepinephrine into the blood vessels, as well as to function as a vasodilator.
- To increase the rates of sodium excreted by the kidneys, as well as the production of urine.
- Causes a decrease in the amount of insulin produced in the pancreas.
- Plays an important role in the digestive system as it induces a reduction in gastrointestinal motility and at the same time protects the intestinal mucosa.
- Moreover, causes a decrease in the activity of the lymphocytes in the immune system.

Dopamine, with the exception of blood vessels, is produced locally in the peripheral systems referred above and acts in the region from which it is released.

Problems in the normal functioning and production of dopamine have been linked to various diseases from time to time including Parkinson's, schizophrenia, addiction as well as attention deficit hyperactivity disorder (ADHD). The basic function of the medicines currently used to treat these diseases is based on the alteration of the effect of dopamine. The most common treatment still used today to reduce Parkinson's symptoms is the use of levodopa, a pure form of L-DOPA. Research has shown that schizophrenia is a cause of altered levels of dopamine activity. For this reason, the antipsychotics used for the treatment of schizophrenia have the essential characteristic of being dopamine antagonists, thereby reducing dopamine action (Moncrieff, 2008). In contrast to schizophrenia, ADHD is due to reduced dopamine levels and activity (Volkow et al., 2009), so dopaminergic stimulants at lower doses are considered a solution for the treatment of ADHD.

Synthesis

The dopamine neurotransmitter is produced in the medulla of the adrenal glands by tyrosine (Seeman, 2009). The precursor molecule of dopamine is L-DOPA which can be synthesized either directly from the tyrosine amino acid or indirectly from the phenylalanine amino acid (Musacchio, 2013). Phenylalanine is the precursor molecule of tyrosine, which, with the help of phenylalanine hydroxylase, is produced in the liver. Both phenylalanine and tyrosine are found in almost every protein that makes these amino acids easy to absorb from food. Like the above amino acids, dopamine is available in many foods, but because it cannot enter the brain through food and perform its neuronal function, it has to be synthesized within the brain. L-tyrosine is produced by L-phenylalanine by the action of the enzyme phenylalanine hydroxylase. L-DOPA is then produced from the amino acid L-tyrosine by the action of the tyrosinase enzyme, an aromatic amino acid hydroxylase. In turn, L-DOPA is converted to dopamine by the effect of the aromatic amino acid decarboxylase enzyme. In addition, dopamine can be used as a precursor for the production of norepinephrine by the action of the dopamine β-hydroxylase enzyme (Figure 3) (Musacchio, 2013).

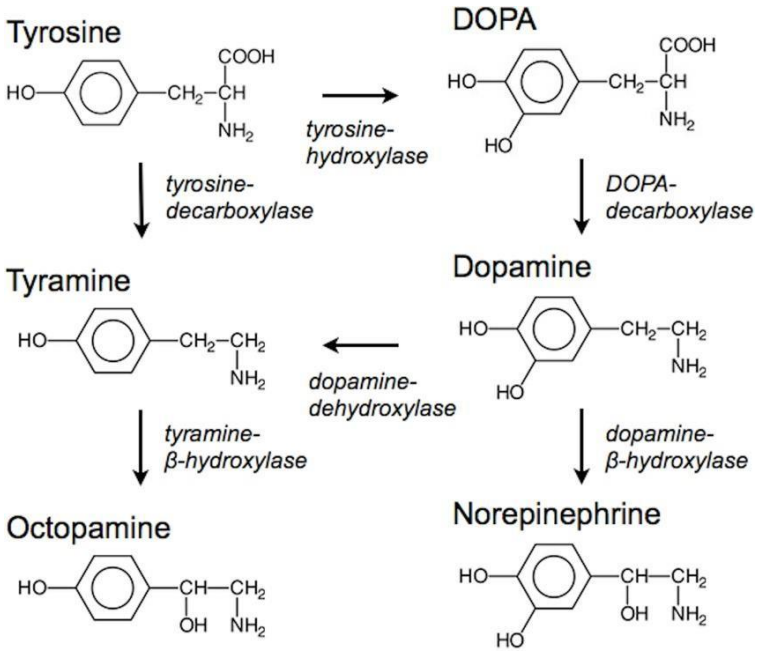


Figure 3. *Biosynthetic pathways for Catecholamines and trace amines (Figure adapted from* http://www.frontiersin.org/files/Articles/1875/fnbeh-04-00163-HTML/image_m/fnbeh-04-00163-g001.jpg)

1.1.2 Dopamine receptors

There are five dopamine receptors known as D1, D2, D3, D4, and D5 (Seeman, 2009). The existence of dopamine receptors was first reported in 1972 where it was observed that dopamine has the ability to stimulate adenylyl cyclase (AC) activity (Brown et al., 1972; Kebabian et al., 1972). Various studies have shown that dopamine can bind to various sites (Cools et al., 1976), thus concluding that there are two populations of dopamine receptors: one that is positively coupled to AC and the other that is independent of AC (Spano et al., 1978). As a result, researchers classified the dopamine receptors into two subclasses. In the first category belong the D1 and D5 receptors, which have a similar structure and sensitivity to drugs and have been called "D1like" receptors. The other category includes the D2, D3, and D4 receptors, which also have a comparable structure, and have been called "D2 like" receptors. D1 receptors occur more frequently in the human nervous system. The second most abundant are D2 receptors, compared to the other receptors (D3, D4, D5) which are in a lower amount in the nervous system (Beaulieu et al., 2015; Romanelli et al., 2009; Jader et al., 1996).

D1-like Dopamine Receptors

The D1-like receptor family includes the D1 and D5 receptors. The D1-like receptors can act by excitation via the opening of sodium channels or inhibition via the opening of potassium channels. The D1 receptors are concentrated in the hippocampus, putamen, nucleus accumbens, olfactory tubercle, and frontal hypothalamus, substantia nigra pars reticulata, caudate, and temporal cortex (Sadock et al., 2009; Grandy et al., 2016). The D5 receptor is expressed in the nucleus of thalamus suggesting that role in pain stimuli (Sadock et al., 2009; Rang, 2003). D1 receptors are involved in locomotor activity, learning, the regulation of the reward system, and memory (Vekshina et al., 2017; Graybiel et al., 1994). In particular, they induce the stimulation of AC activity leading to the production of cyclic AMP which acts as a secondary messenger (Figure 4). D1 receptors are directly linked to several neuropsychiatric conditions as they are involved in signal transduction pathways, activating the phospholipase C which results in the release of intracellular calcium. The calcium released has the ability to activate certain types of proteins such as calcium-dependent protein kinase C (PKC), but also through exocytosis it has the ability to regulate neurotransmitter release (Ha et al. 2012). D1

receptors can regulate the electrochemical gradient through Na + / K + -ATPase pump ions. Activation of D1Rs has been shown to inhibit Na + / K + - ATPase pumps through the protein kinase A (PKA) and PKC signaling pathways in the striatum (Nishi et al., 1999; Gomes et al., 2002) and in the kidney (Missale et al., 1998).

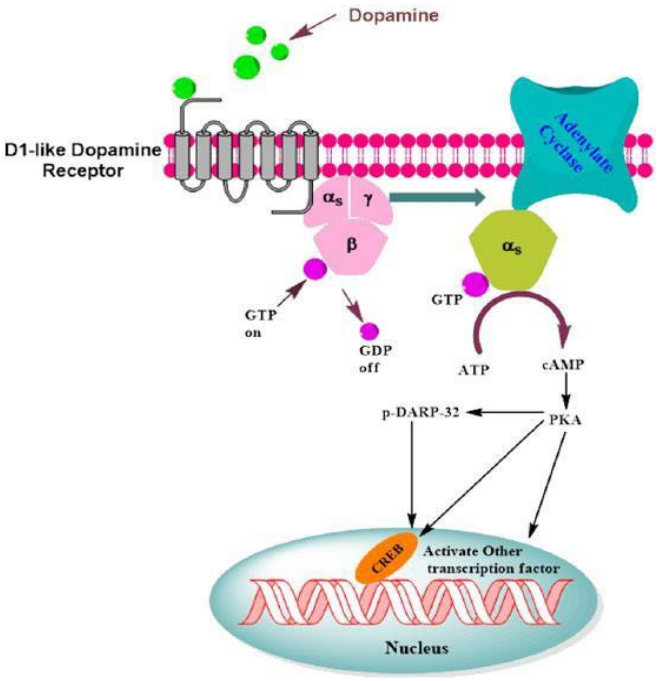


***Figure 4**. Schematic representation of dopamine "D1-like" receptor' mechanism (Adapted from Akanksha et al., 2018)*

D2-like Dopamine Receptors

The D2, D3 and D4 receptors form the D2-like family. The main role of D2-like receptors is normally the inhibition of the target neuron. D2 receptors are found at high concentrations in the septum, basal ganglia, and ventral tegmental area. The D3 receptors induce positive regulatory effects of dopamine on neuro tension production. The D4 receptor homology with D2 and D3 is 41% and 39%, respectively, and are found in the hippocampus and frontal cortex (Malenka et al., 2009; Nestler, 2014). Activation of the D2-like receptor family causes inhibition of AC activity and inhibits the production of

cAMP and PKA levels as it is shown in Figure 5 (Vekshina et al., 2017; Ben-Jonathan, 1985). D2 receptors are arguably the most important postsynaptic receptors that mediate extrapyramidal and behavioral activity. Most drugs used as antipsychotics block D2 receptors. D3 and D4 receptors are found more frequently in the limbic area of both human and rat brain. The function of these receptors is of great interest as they have been found to have a high affinity for second-generation antipsychotic drugs such as clozapine. These findings are very important as they indicate that the D3 and D4 receptors present in the human brain are capable of mediating the antipsychotic effects of many typical and atypical antipsychotic medications. The presence of these receptors in the limbic area of the brain can lead to the development of neuroleptics that specifically target these regions (Malenka et al., 2009) (Table 3).

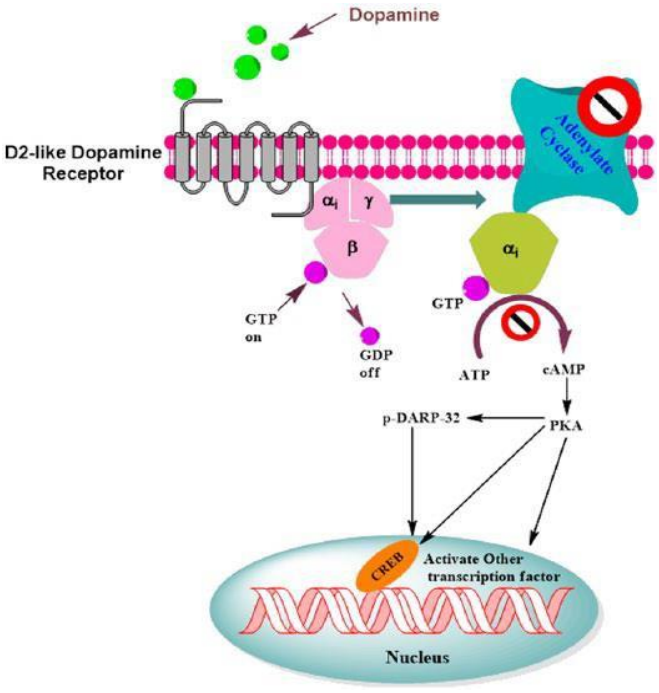


Figure 5. *Schematic representation of dopamine "D2-like" receptor' mechanism (Adapted from Akanksha et al., 2018)*

***Table 3**. Dopamine receptors, location, mechanism, and functions*

Receptors	Location	Mechanism	Function	
D1	Found in high concentration in mesolimbic, nigrostriatal and mesocortical areas, such as substancia nigra, olfactory bulb, nucleus accumbens, caudate, putamen, striatum, Expressed in low level in cerebellum, hippocampus, thalamus, hypothalamus, and kidney.	Increased intracellular level of cAMP by activated adenylate cyclase	• Voluntary movements • Regulate growth and development, • Regulations of feeding • Affect • Attentions • Reward	• Sleep • Impulse control • Reproductive behaviors • Working memory • Learning • Control of rennin in kidney.
D2	Expressed in high levels in as substancia nigra, olfactory bulb, caudate, putamen, ventral tegmental area(VTA), nucleus accumbens Found in low level in hypothalamus, septum, kidney, cortex, heart, blood vessels, adrenal glands, gastrointestinal tract, sympathetic ganglia.	Increased intracellular level of cAMP by activated adenylate cyclase	• Involved in working memory • Reward-motivation functions • Regulate blood pressure • Regulate locomotion - presynaptic receptors • Gastrointestinal motility	• Vasodilatations • Inhibit locomotion and post synaptic receptors • Renal functions • Activate locomotion
D3	Expressed only in CNS and it is not found outside the CNS. Found in olfactory bulb, nucleus accumbens.	Adenylate cyclase↓	• Involved in endocrine function cognitions • Emotions • Regulations of locomotor functions • Modulates endocrine functions	
D4	Substancia nigra, hippocampus, amygdala, thalamus, hypothalamus, kidney, frontal cortex, heart, blood vessels, adrenal glands, gastrointestinal tract, sympathetic ganglia, globus pallidus, Lowest receptor concentration found in CNS than all dopamine receptors.	Adenylate cyclase↓	• Regulations of renal functions • Gastrointestinal motility • Vasodilatations • Blood pressure • Modulations of cognitive functions	
D5	Substancia nigra, hypothalamus, hippocampus, dental gyrus, kidney, heart, blood vessels, adrenal glands, gastrointestinal tract, sympathetic ganglia.	Adenylate cyclase↑	• Involved in pain process • Affective functions • Endocrine functions of dopamine	

1.1.3 Dopamine agonist and antagonist

Agonists

An agonist is a chemical compound which is binding to a receptor and activates it, driving to a final result similar to the reaction that is usually caused by the binding of the endogenous substance. Therefore, a neurotransmitter agonist will elicit a similar response to the receptor as the transmitter. Drugs that act as agonists have the ability to activate the receptors both directly and indirectly. As for drugs that act as direct agonists, they are further categorized and characterized as partial, full or even inverse agonists.

Agonists acting as direct have the ability to induce the same effect as a neurotransmitter when they bind to receptors that are usually located in either the synaptic or postsynaptic neurons (Richard et al., 2009). Opiates, which are used to relieve pain and also to cause euphoria, are a characteristic example of direct agonists (Ronsley et al., 2020), with the main representatives of this class being morphine, methadone, oxycodone, and heroin. Another direct agonist found in large quantities in tobacco is nicotine, which has the ability to bind to nicotinic acetylcholine receptors (Flore et al., 2013).

Agonists with indirect action have the potential to inhibit the reuptake of neurotransmitters or stimulate their release, leading to increased binding of neurotransmitters to their target receptors (Miller, 2011). A typical example of an indirect agonist is amphetamine, which, as an indirect agonist of the dopamine, serotonin and norepinephrine receptors, acts either to induce neurotransmitter release or to prevent reuptake from the synaptic cleft (Eiden et al., 2011; Richard et al., 2009).

Some medical drugs act as dopamine agonists and can be used for treatment of hypodopaminergic (low dopamine) conditions, Parkinson's disease (PD) (Jankovic et al., 2020), attention deficit hyperactivity disorder (ADHD), certain pituitary tumors (prolactinoma) (Allard et al., 2020), and may be useful for restless legs syndrome (RLS). Some examples of those agonist are listed to the Table 4.

Table 4. *Dopamine agonists and their major clinical uses*

Dopamine Agonist	Major Clinical Uses
Apomorphine	PD, erectile dysfunction
Bromocriptine	, hyperprolactimenia, type 2 diabetes
Cabergoline	Pituitary tumors, hyperprolactinemia
Fenoldopam	Hypertension
Pramipexole	PD, RLS, bipolar disorder, depression
Pergolide	PD
Ropinirole	PD, RLS
Rotigotine	PD, bipolar disorder, depression
Amphetamine	ADHD, narcolepsy, obesity
Bupropion	cessation, nicotine addiction, depression
Lisdexamfetamine	ADHD, binge eating disorder
Methylphenidate	ADHD, narcolepsy
Methamphetamine	stances to treat ADHD and obesity

Antagonists

As an antagonist, it can be called a compound that has the ability to bind to the receptor, thereby preventing its activation. Alternatively, it may also be referred to as a receptor inhibitor, because it has the ability to bind to the receptor and essentially inhibit the effect of the agonist which will lead to activation of the receptor and therefore to the production of a biological reaction. Antagonists are divided into the competitive antagonist and irreversible antagonists.

As mentioned above, there are two types of antagonists. When the antagonist binds to the receptor at the same position as the agonist but with competitive action, it is called a competitive antagonist. As the agonist and antagonist compete for the same site, an increase in antagonist concentration will lead to a decrease in the biological response of the receptor due to the decreased ability of the agonist to bind to the receptor. The biological response of the receptor may also be completely inhibited if the antagonist concentration is high in relation to the agonist. However, this situation can be reversed if the concentration of agonist is increased relative to the concentration of antagonist. It should be noted that when a competitive antagonist is present, in order for the receptor to produce the same biological response as in the absence of the antagonist, then the concentration of the agonist must be relatively increased (Gonsai et al. 2018).

The second class are the irreversible antagonists which bind to the receptor very powerfully, forming covalent chemical bonds with the receptor, and making the agonist

unavailable for binding to the receptor. When the irreversible antagonist appears in relatively high concentrations, the number of the receptors that are available for the agonist to bind on may be so low, that despite the presence of aloft concentrations of the agonist, the elicitation of the physiological response is not the same (Goeders, 2014).

Most dopamine antagonists are used in treating bipolar disorder, stimulant psychosis, schizophrenia, nausea and vomiting (Gan et al., 2019) (Table 5).

Table 5. *Dopamine antagonist and their major clinical uses*

Dopamine Antagonist	Major Clinical Uses
Amisulpride, Aripiprazole, Olanzapine, Ziprasidone, Quetiapine	Schizophrenia, bipolar disorder, depression
Chlorpromazine, Clopenthixol, Clozapine, Promazine, Droperidol, Haloperidol	Schizophrenia
Domperidone, Metoclopramide	Nausea
Risperidone	Schizophrenia, bipolar disorder
Prochlorperazine	Schizophrenia, nausea
Sulpiride	Schizophrenia, depression

1.2 Biosensors

The first biosensors were developed in the second half of the 20th century. In 1956, Professor Leland Clark discovered the first oxygen electrode and then in 1962 presented the first biosensor, which was an electrochemical (enzyme electrode) glucose sensor. The first enzyme-based sensor was described by Updike and Hicks in 1967. At the same time, Guilbault and Montalvo built the first potentiometric biosensor in 1969, and in the 1980s, fiber optic sensors were developed to detect in vivo gas in the blood. In 1983, the first surface plasmon resonance immunoassay (SPR) was invented, followed in 1984 by the first amperometric biosensor, which used ferrocene in combination with glucose oxidase to detect glucose in the blood (Newman & Turner, 2005). Since then, a lot of studies have been accomplished regarding the design of biosensors for a variety of applications.

Biosensors are analytical devices capable of providing quantitative or semi-quantitative information using a biological identifier that is in direct contact with a suitable transducer. The biosensor responds to a physical or chemical stimulus (heat, sound, electric charge, etc.) and converts the biological response into a measurable external signal.

Biosensors consist of two main parts (Figure 6):

- The receptor which consists of a specific biomolecule (protein, antibody, DNA, cells) that reacts specifically with an analyte (antibody, ions, heavy metals, gases, etc.). The biomolecule has the ability to recognize both the presence and concentration of a specific analyte in solution,

- The transducer capable of converting the response from either a biological or biochemical analyzer into a measurable electrical signal (Figure 1.9). The size of the electrical signal produced is equivalent to the analyte concentration (Mohanty & Kougianos, 2006).

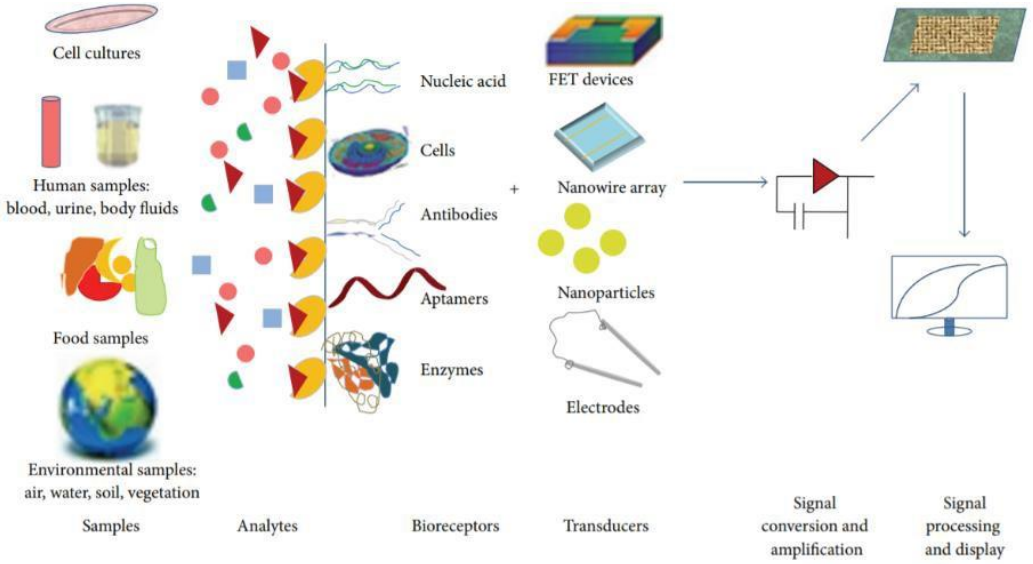


Figure 6: *Schematic diagram of biosensors components (Grieshaber, 2008)*

1.2.1 Types of biosensors

The biosensors classification is based on the signal transducer that is used, the nature and mode of action of the biomolecule, and the immobilization method. Based on the signal transducer, biosensors are distinguished in optical (absorption, fluorescence, chemiluminescence), electrochemical (amperometric, potentiometric), thermal (emission measurement and heat absorption) and gravimetric (mass changes) (Eggins, 1996).

Optical biosensors are quite widespread and are based on the change in optical properties (in absorption or fluorescence) of the biomolecule recognition element when detecting the analyte (Damborský et al., 2016).

Electrochemical biosensors are the most broadly used biosensors worldwide. The signal transducer is electrochemical and is normally an electrode. The conversion of the signal from electrochemical to measurable electric is easy and of low cost (Lima et al., 2018). The electrochemical signal transducers are distinguished in potentiometric and amperometric. Potentiometric biosensors measure the oxidative / reduction potential of an electrochemical reaction, recording the potential flowing through an electrochemical element under zero-current conditions. Amperometric biosensors are based on the immobilization of the bioassay element on the solid state electrode surface (platinum, graphite, carbon, metal oxides, and polymeric printed electrodes) which constitutes the biosensor transducer. Under a steadily applied potential difference, the electrode measures the current generated during a redox reaction. During the reaction, the biological element produces a current proportional to the concentration of the analyte (Bhalla et al., 2016).

Additionally, there are thermal biosensors which work by measuring temperature from the heat absorbed or released when the analyte is recognized by the biomolecule (Gorton, 2005).

Gravimetric biosensors generate a signal due to the change in mass. Most gravimetric biosensors use piezoelectric quartz crystal as surface acoustic wave devices or as resonating crystal devices. When a biomolecule binds, a measurable signal is generated due to the change in mass.

Finally, based on the type of biomolecule recognition element, the biosensors are distinguished in enzyme- (immobilized enzymes and proteins), DNA biosensors (nucleic acids), cellular biosensors (immobilized cells or tissues), immunosensors (an immobilized antibody or antibodies coupled with an enzyme or pigment) and microorganism-based biosensors (Zhang et al., 2019).

1.2.2 Cell-based biosensors

Living cell-based biosensors are techno-scientific systems that use cells as sensors to detect the status of the cellular environment and physiological parameters. The innovation of those biosensors is that unlike other types of biosensors containing only living-extracted elements, they use live cells as receptors. This type of biosensor consists of (a) living cells, the biological identification element, that acts as the primary signal transducer element and used as the primary element for the collection and transmission of signals, and (b) the secondary transducer element that convert those responses in electrical signals (Gupta et al., 2019).

During the last 15-20 years, cell biosensors became the epicenter in research in the area of biosensors and bioelectronics, due to their ability to extract information from active biological analyzers. These biosensors have the advantage of selectivity, rapid response and high sensitivity and are applicable to a variety of fields like environmental monitoring, biomedicine, and pharmacological testing (Inda et al., 2019; Kintzios 2006).

A cell biosensor consists mainly of two parts: a) living cells or neural networks which have been cultivated on the surface of the signal transducer element and b) a transducer for chemical detection or potential detection. When cells interact with a stimulus, they produce changes in molecules or ions, changes in potential or changes in impedance due to cell metabolism, etc. A secondary transducer element can detect these responses and convert them into electrical signals (Gupta et al., 2019)

Cell-based biosensors have the advantage of increased stability, high biocatalytic activity, and their main feature is their ability to provide relevant cellular information in response to the sample and finally measure its activity. In other words, cell-based biosensors that perform functions such as cells, not only detect the presence of an

element but are able to respond in a way that senses the physiological effect of the sample on cells (Bickerstaff 1997).

In addition, the need for biosensors with a lifetime of weeks or months excludes the use of older cells, which requires time to collect and prepare the cells. Cell-based biosensors also provide a more complex sensor system which in combination with their economical construction makes them an attractive method.

However, as with other biosensors, they have limitations in their sensitivity and reliability due to environmental changes (such as pH and temperature). Also, due to the presence of various enzymes and receptors in the tissues, cell-based biosensors have low selectivity because they are likely to respond to different substances than desired. A further disadvantage of biosensors based on plant cells or microorganism cells is the diffusion of the sample through the cell wall, resulting in a slow biosensor response compared to the immediate response of enzymatic biosensors (Rainina et al. 1996).

1.2.3 Mammalian based biosensors

Using mammalian cells for biosensors applications was first undertaken by Giaever and Keese in 1993. They used for the first time an “electrical cell-substrate impedance sensing” (ECIS) system for detecting changes in cell morphology. Animal cells in biosensors are being mostly used in toxicological/environmental analyzes and in food control (Gupta et al., 2019). In Table 6 major developments in this area, categorized cell lines, and measurement techniques are presented (Kintzios & Banerjee, 2015).

Table 6. *Examples of biosensors based in mammalian cells used to food control (adapted from Kintzios & Banerjee, 2015).*

Cellular material	Measurement technology	Target(s)	Detection limit	Quantitative determination	References
Cardiac cells	Electrophysiological	Pyrethroid pesticides	Not determined	No	Natarajan et al., (2006)
Vero and N2a cells	Electrophysiological (bioelectric recognition assay)	Organophosphate and carbamate pesticides	ppt	Yes	Flampouri et al., (2010); Mavrikou et al. (2008)
B lymphocyte Ped-2E9	Colorimetric	Foodborne pathogens and their toxins	103 CFU/ml (listeria), 10–40 ng (toxin)	Yes	Banerjee, Franz, and Bhunia (2010)
Two human and mouse hepatoma cells, breast cancer cells	Bioluminescence	Polyhalogenated aromatic hydrocarbons (PAHs)	fM	No	Murk et al. (1996)

While there are limited biosensor systems based on animal cells that are used commercially, this technology is often referred to in the international scientific literature and accounts for a significant number of cell technologies (Inda et al., 2019). On the other hand, cell biosensor technology appears to be attractive in terms of intellectual property protection and the detection of pesticide residues in plant and other chemical residues, and patent applications in food microbiology cannot be considered negligible. In addition, 8% of European social funds funded food safety projects since 2000, with the one-quarter of these projects targeting cell-based biosensors and more specifically the use of mammalian cells as biological recognition elements (Kintzios & Banerjee, 2015).

1.2.4 Technologies and challenges

Biosensors based on mammalian cells due to reduced vitality and the resulting limited conservation potential have attracted the interest of biosensors experts. Also, while cells have the ability to respond in a physiologically related manner to various analyzers, the response is often not always as reproducible with respect to the signal of

origin. In other words, the size of the response may vary to the extent that the lack of reproducibility will hinder the practical application of a cellular biosensor.

Natarajan et al. (2006) developed a high-performance method for the detection of pyrethroids using a multi-electrode system for extracellular measurements of cardiac muscle cells cultured on a microelectrode array (MEA). The activity of the cells extracellularly produced a potential range from 100 μV to 1200 μV. Pyrethroids such as tetramethrin, α-cypermethrin, and teflutrin, tested, caused alterations in the electrophysiological properties of cardiac muscle cells with a similar pattern, i.e. reduced frequency and pulse intensity.

Banerjee and Bhunia (2009) used Ped-2E9 lymphocytes previously immobilized to detect pathogens such as *Listeria monocytogenes*, Bacillus, Vibrio, and toxins such as hemolysin, phospholipase C, and enterotoxin from Bacillus. The detection limits for the toxins were 10-40 ng in 2 hours, while for *L. monocytogenes,* 10^3-10^4 CFU / mL in 4-6 hours. Hepatoma cells which are known to express the aromatic hydrocarbon receptor (AhR) were used to detect dioxins. In particular, they created transformed cells, inserting into them a plasmid carrying the dioxin gene and the luc gene encoding luciferase. The limit of detection was 5 x 10-12 mol / L 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). The biosensor also responded to other chemicals that had a similar chemical structure to dioxins (Murk et al., 1996; Michelini et al., 2006).

While the applications of cell-based biosensors developed very quickly, the theoretical background of their function has not yet been clarified in many cases. There are disagreements about the signal emitter mechanism and the interpretation of the measured and expected results. At the same time, the development of cell-based biosensors means the simultaneous conducting of extensive research. This is one of the problems of commercialization of cellular biosensors. Although there are some research projects with relatively high success rates, cell biosensors still have some drawbacks. These are the following:

- The response of some types of cellular biosensors is slower than enzyme sensors.
- The time it takes, after their use, to return to their original state is usually longer.
- Enzymes are contained in cells, so in order to obtain high selectivity, cells must be treated with extreme caution.

1.2.5 Type of cell-based biosensors - Applications

Electrically active cells or tissues can be crosslinked with microelectrodes that allow the capture of extracellular signals associated with cellular responses or whole tissue responses. This interaction is categorized into three groups:

- Direct interaction of microelectrodes with the membrane of cell membrane
- Interplay with analyte components present in the extracellular microenvironment
- Interplay with other targets' parts, like solid colloid and solution concentrated in uterine pores (immobilized cell biosensors) or culture solution (suspension of cell biosensors).

Currently, the secondary transducer element used in cellular biosensors mainly comprises field-effect transistors (FET), light-addressable potentiometric sensors (LAPS), Electric Cell-substrate Impedance Sensing (ECIS), patch clamp, quartz crystal microbalance (QCM), surface plasmon resonance (SPR), etc.

The general principle of operation of electrode-based methods is the following: an electrode consisting of conductive wire (e.g., silver, gold or platinum) is placed in a pipette filled with an electrolytic solution. With this system, when the tip of the pipette comes into contact with the biological material, a conductive bridge is created, through which a current or potential recording device can be connected. In addition to the measuring electrode, the reference electrode is at a steady current or potential value. Two electrode pairs are usually used to measure current or potential after external current or potential application (amperometry - voltammetry).

FETs have the ability to measure changes in load accumulation on their surface (Kaisti, 2017). Therefore, if the surface is modified with special selective materials, FETs is converted into a device sensitive to changes in the surface potential. That is, FETs can be converted to ISFET (ion-sensitive field-effect transistor). The potential is proportional to the concentration of the external ions or generally the charged molecules, against which the particular ISFET is selective (Parizi et al., 2017). Also, FETs can be converted into a biosensor based on a field-effect transistor (BioFET) (Sakata, 2019), where due to the binding of molecules to the biosensor, changes in surface potential are induced (Figure 7). In particular, when charged molecules bind to the dielectric material of the FET gate, they cause changes in the conductivity of the channel due to the change in the

charge distribution of the underlying semiconductor material. Cell-based FET sensors study changes in membrane potential and changes in ion concentration. If there is a change, an increase in the surface potential of the dielectric layer is observed, causing the density of the moving electrons to change. Cell-silicon interaction induces current synchronization across the inverse layer (Geisler et al., 2006).

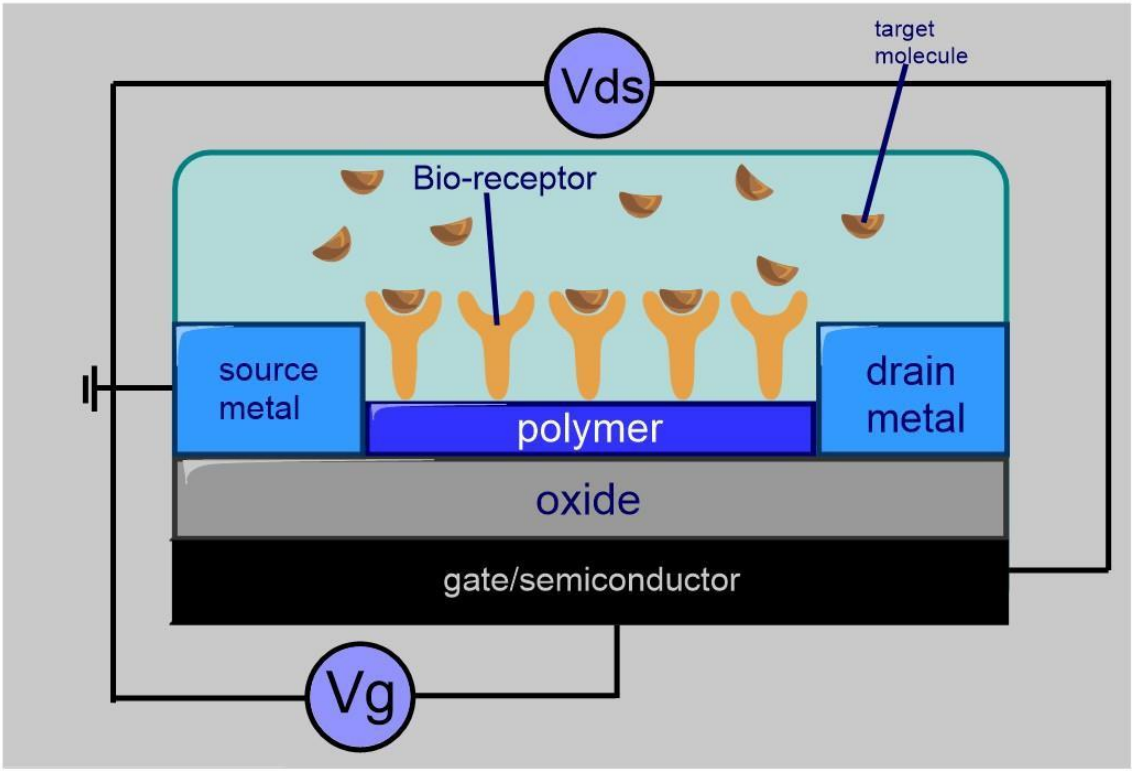


***Figure 7**. Schematic representation of a BioFET (Figure adapted from https://commons. wikimedia.org/wiki/ File:BioFET.jpg)*

A light-addressable potentiometric sensor (LAPS) is a semiconductor device that uses light and was proposed for the first time by Hafeman et al. in 1988 (Zhang et al., 2001). Compared to other chemical sensors, LAPS has several advantages. They have a flat surface that does not require various structures, nor any type of wiring or passivation. Also, the LAPS layout facilitates obtaining a spatial map that illustrates the distribution of the ion concentration in the sample. This analysis is an important factor and is basically determined by the lateral diffusion and beam size of the photocarrier present on the semiconductor substrate (Liang et al., 2019). With the illumination of portions of

the semiconductor surface, electron-hole pairs are produced and photocurrent flows. Based on LAPS, in 1990, the first cell-sensing Cytosensors microphysiometer from Molecular Device was launched. This may detect small changes in the oxidation in the extracellular cell density microenvironment. Cells are cultured in the microenvironment of the sensor (Figure 8). Variants of specific molecules identified when recording the cell's biological responses. The Cytosensors microphysiometer differs from most analyzers and this is because it has the ability to detect the effect of the analyzer on the cells rather than specifying the characteristics of the analyzers (Hafner, 2000). In most cases, acidic products of cellular metabolism are associated with ATP consumption. During significant metabolic processes (e.g. glucose metabolism, aminophenol and fatty acids), induced proton release in the microenvironment causes a change in pH at the extracellular environment. Cytosensors microphysiometer has the ability to measure so minor changes and can nominate changes in cellular metabolism (Nash et al., 2014).

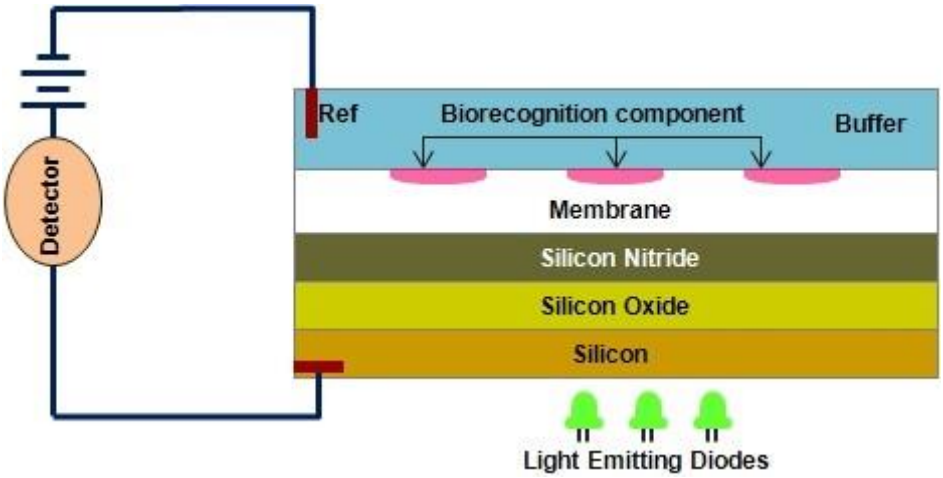


Figure 8. *Schematic diagram of LAPS with biorecognition components bound into the membrane (Ahmed Touhami, 2014)*

ISEs record the potential that develops on the surface of a membrane (solid or liquid) as a function of a reference system (semiconductor with known and constant potential). Usually, on the membrane/solution surface an exchange or ion exchange equilibrium occurs. The potential developed depends mainly on the change in free energy associated with mass transfer across the membrane/analyte surface. ISEs are mainly

used in cases where knowledge of the concentration of ions in an aqueous solution is required, such as in analytical chemistry.

ECIS has been used in various techniques (Gelsinger et al., 2019), such as electrical spectroscopic impedance, to investigate the bioelectric properties of cell membranes and the mechanisms of response to chemical or electrical impulses as shown in Figure 9. The impedance systems need some time to give a response, which is nevertheless faster than traditional biological assays in cell culture. This has nothing to do with the speed of impedance spectrometry but with the time it takes for the toxicant to interact with the cell system to produce measurable results.

Brennan et al. (2012) managed to improve both testing speed and the ability to maintain the cellular bioassay element using trout epithelial cells (RTgill-W1) in combination with ECIS.

Curtis et al. (2009) wanted to evaluate the response of various cell lines to different substances, thus they obtained 10 different mammalian cell lines and, with the help of an impedance biosensor, evaluated the response of the cell lines to aldicarb and sodium pentachlorophenate. Data were collected over an hour and evaluated within 15 minutes, thus reducing the duration of the test. At the same time, the cells were compared for their ability to form stable monolayer structures as well as for their ease and high impedance. The HUVECs, Vero, IgH-2, and BLMVECs cell lines were selected for the screening experiments because they presented a better effect on the ability to form stable monolayers on ECIS electrodes. The Vero and HUVECs cell lines showed the highest sensitivity to aldicarb and sodium pentachlorophenate with an effective concentration around 1 and 0.38 mM, respectively, for both toxicants. The lifetime of the monolayers in ECIS electrode arrays was 37 days. These results lead to a satisfactory cell storage life which can be increased by the use of an enclosed fluidic biochip.

Today there is a remarkable number of biosensor-based methods that measure changes in electrophysiological interactions. Some of them use cells, others use electrical properties assays or immobilized elements. The main version and the most common electrophysiological method of measuring interactions are where the biosensor includes the cellular part connected to the end of a microelectrode and communicates with a transformer that measures electrical current in picoampere. According to this method (patch-clamp), a single cell or part of the cell membrane comes in direct contact with an

electrode. Normally a voltage is applied to this segment and then the change in the value of the membrane current in picoampere is evaluated. The patch-clamp method (Brown, 2016) is extensively used in the fields of electrophysiology and neuroscience (Figure 10). Nevertheless, this technology has some unavoidable restrictions like the low rate of performance. It is very difficult to apply to drug detection and cellular communication of nervous networks.

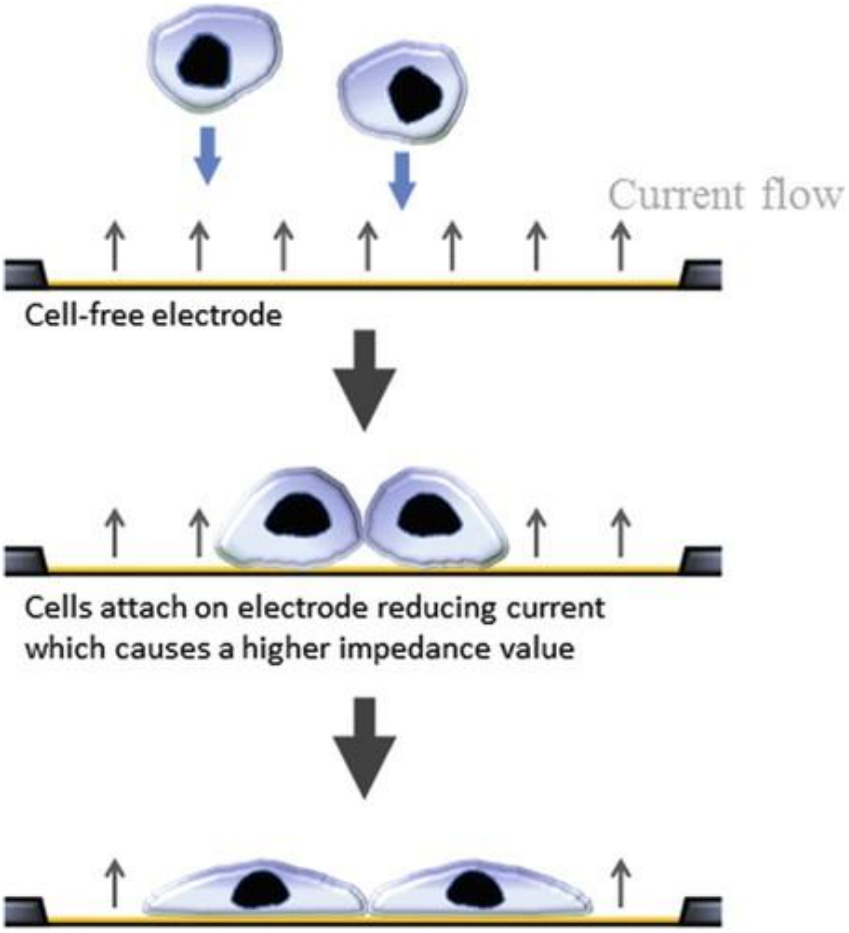


Figure 9. *Measurement of Cellular Behavior by Electrochemical Impedance Sensing (Figure adapted from Simin Öz et al., 2017)*

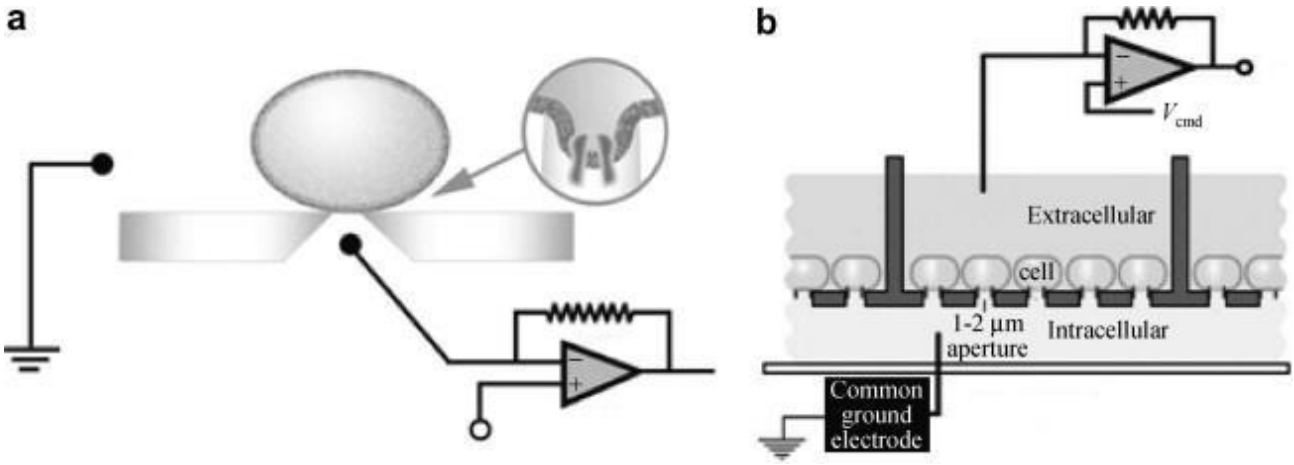


Figure 10. *Patch clamp chip. (a) Scheme of measurement principle; (b) patch clamp array (Adapted from Chen et al., 2008)*

Eventually, the immune system is one of the foremost complicated biological systems. It protects against diseases by recognizing and reacting to antigens with great sensitivity and selectivity. A large range of immune cells was studied in order to investigate the possibility that the immune cells will be used as sensing components in cellular biosensors. As an example of cellular bioassay biosensors maize cells and B cells are used and have provided promising applications for the detection of pathogens. Simultaneously, these cell biosensors could be utilized in studies that are fundamental to the cellular interactions of the system and different areas of biology.

1.2.6 Bioelectric recognition assay (BERA) and Molecular identification through membrane engineering (MIME)

In the last nineteen years, a system of measuring the change in electrical potential (or other electrical properties) (Kintzios et al., 2001) of a single cell membrane or single-cell segment but at the tissue level has been developed. BERA is a method for detecting viruses and other bioactive substances based on the measurement of changes in the electrical properties of a cell group properly immobilized within a gel so as to maintain normal cellular functions upon interaction with the viruses under detection. It is possible to detect human and plant viruses in a specific, fast (less than 3 minutes) way, remarkably

reproducible and economical. The sensitivity of BERA detection (0.001-0.1ng) is comparable to previous immunological, cytological and molecular techniques.

Six generations of BERA cell biosensors have been developed until now. In the first generation, the biosensor contained immobilized cells in 0.8% agarose and was in the form of a 15 mL tube (Kintzios et al., 2001). The second generation also had the form of a tube and the cells were immobilized on agarose, except that it had a much smaller volume (1 mL), which required a smaller amount of materials. The 3rd and 4th generation BERA cell biosensors were in the form of a flat layer and in the third generation the cells were immobilized in calcium alginate "peel", and in the fourth generation a conductive calcium alginate matrix was formed with wells at the bottom of which the electrodes were inserted and the cells were applied into these wells. The third generation BERA biosensors are suitable for the study of cytotoxicity (Kintzios et al., 2006) and the fourth generation for the study of cell communication and interaction with various substances. Finally, the 5th and 6th generation BERA biosensors were in the form of beads, immobilized cells in calcium alginate (Moschopoulou and Kintzios 2006). The difference between these two generations is that in the 6th generation biosensors the immobilized cells are membrane-modified (MIME) using membrane engineering technique through the electroporation of molecules in the cell membranes, for the purpose of the selectiveness of the system.

Cell biosensors are considered to be an attractive tool due to their high sensitivity that in some cases it may even reach the level of recognition of a single molecule. This theoretical hypothesis relates to the complex interaction that has occurred as a result of the co-evolution of cells with numerous molecules in their surroundings to the extent that it permits cells to respond to molecules and organisms with accurate and reproduced ways. However, this advantage also comes with an evolutionary disadvantage as cells have the ability to react in a similar manner to a large number of molecules, making the cell biosensors less selective. This is also the major problem we face in toxicological methods since on the one hand, the sensors detect the toxic agent but on the other, it is very difficult to determine the nature of the toxic analyzer (Van der Lelie et al., 1997; Riska et al., 1999).

In recent years, due to the growing demand for cells with increased selectivity, cell transfection methods have been relatively successfully developed. These methods are

primarily based on the uptake by cells of a foreign DNA usually derived from human B cells, thereby leading to the expression of bound receptor membranes (Banerjee & Bhunia, 2009; Rider et al., 2003). One well-known application based on state-of-the-art technology developed by MIT's Lincoln Lab, is the CANARY system. This method has been used to detect *Yersinia pestis* and other pathogens (Rider et al., 2003) with a disadvantage to both the undesired alteration of the cellular phenotype and the lack of stability.

A technology-based on membrane engineering has recently been developed and may well be considered as another approach to cellular modification. The method is based on the insertion, by electroporation, of a large number of receptor molecules (antibodies, enzymes, etc.) on the cell membrane, increasing their selectivity in recognizing target analytes (Moschopoulou & Kintzios, 2006; Moschopoulou et al., 2012). The methodology is based on measuring the change in membrane potential caused by the binding of the target molecule to the receptors previously inserted into the cell membrane (Figure 11). In the beginning, the cell membrane potential is stable due to the ions flowing through the ion channels (a). Subsequently, and after the target molecule binds to the receptor, its structure changes, resulting in its molecular charge being displaced within the cell membrane (b). This has, as a result, a large number of ions to concentrate on one side of the membrane and leading to its hyperpolarization. Opening the ion channel creates an ionic current that can be measured as a corresponding current (c).

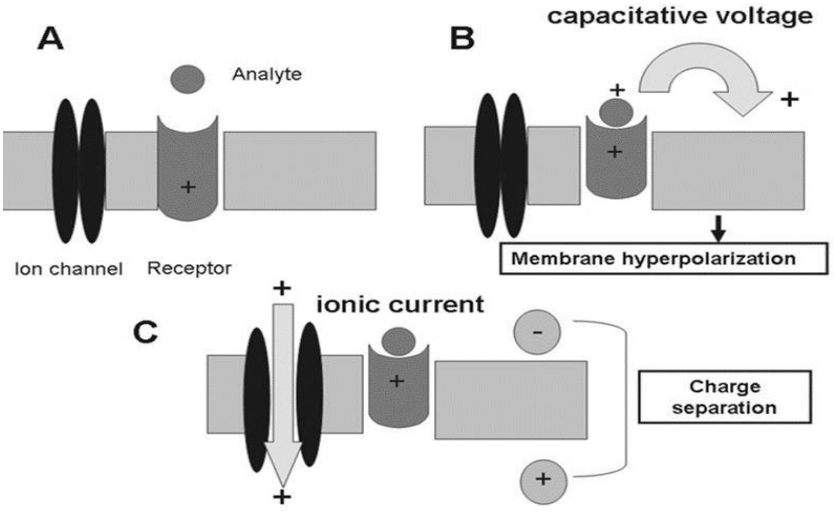


Figure 11. *Working concept of MIME*

Sixth generation biosensors have been developed for various applications such as detection of phytopathogenic and animal viruses, plant protection products and molecules involved in food safety (Mavrikou et al., 2008; Moschopoulou et al., 2008; Perdikaris et al., 2009; Flampouri et al., 2010; Varelas et al., 2010; Apostolou et al., 2014; Mavrikou et al., 2018; Apostolou et al., 2020; Mavrikou et al., 2020).

Chapter 2

Assessment of *in vitro* dopamine-neuroblastoma cell interactions with a bioelectric biosensor

Part of this chapter has been published in the form of original research article with the following data: Apostolou, T., Moschopoulou, G., Kolotourou, E. & Kintzios, S. (2017) Assessment of in vitro dopamine-neuroblastoma cell interactions with a bioelectric biosensor: perspective for a novel in vitro functional assay for dopamine agonist/antagonist activity, Talanta, 170, pp. 69-73.

2.1 Abstract

Existing dopamine receptor binding assays do not quantify the total cellular response to this monoamine neurotransmitter or potential agonist/antagonist molecules *in vitro*. In this work, we report the development of a completely new concept of analysis, in order to study, *in vitro*, the interaction of dopamine with dopamine receptor-bearing nerve cells. The idea is based on the extremely rapid measurement of changes observed in the electrical properties of cultured mouse neuroblastoma (N2a) cells. Analyzing the experimental data, a relatively close relationship was observed between dopamine concentration and cell membrane potential. At low concentrations of dopamine (nanomolar) membrane depolarization was observed, while at higher concentrations (micromolar) cell membrane hyperpolarization was observed. The use of eticlopride, a dopamine D2- receptor antagonist, resulted in concentration-dependent membrane depolarization. In addition, a significant attenuation of cell membrane hyperpolarization was observed when cells were treated with sodium chloride. As determined by cyclic voltammetry, the bioelectric response to dopamine was closely correlated with the pattern of dopamine overflow by N2a cells. The novel approach was additionally used to evaluate the dopaminergic activity of chaste tree extracts (*Vitex agnus-castus*) as well as the activity of two well-known antipsychotics, haloperidol, and olanzapine. This new concept offers many promising prospects for the development of an advanced system for the rapid functional characterization of neurotransmitters agonists and antagonists.

2.2 Introduction

One of the most important aspects of brain function is dopamine signaling (DA). Many side effects of major diseases and pathologies, as well as the basis of their treatment, are based on the pharmacological modification of the response of the various dopamine receptors and its subtypes (Abraham et al., 2014). Without diminishing the importance of receptor affinity studies, another area that is equally important in neuropharmacology research is the functional assays for testing the activity of different drugs that have agonistic or antagonistic activity in relation to DA. Although they often use only a small percentage of the total receptor population in the biological sample system, they provide increased and more accurate information on the efficacy and potency of the identified drugs, especially at very low concentrations of agonists (Kenakin, 2009).

In recent years, mammalian cell-based biosensors became the epicenter of research in the area of biosensors and bioelectronics, due to their ability to detect information from active biological analyzers. These biosensors have the advantage of selectivity, rapid response, and high sensitivity and are applicable to a variety of fields such as clinical analytical science. Cell-based biosensors have the advantage of increased stability, high biocatalytic activity, and their main feature is their ability to provide relevant cellular information in response to the sample and ultimately measure its activity. In other words, cell-based biosensors that perform functions such as cells not only detect the presence of an element but are able to respond in a way that senses the physiological effect of the sample on cells (Kintzios, 2007; Banerjee et al., 2013). In addition, biosensor-based methods that measure changes in electrophysiological interactions (such as resistance or membrane potential) are quite popular because of their speed and ease of use. Among these techniques, a system of measuring the change in electrical potential (or other electrical properties) of a single cell membrane or single-cell segment has been developed at the tissue level. Bioelectric Recognition Assay (BERA) is a method for detection of bioactive substances based on the measurement of electrical properties of a cell group, and to date has been used in many applications, such as recently, for the determination of peroxide levels by PC12 cells in vitro (Moschopoulou & Kintzios, 2015). Although many electrophysiological methods such as single-unit

recording (Wuttke et al., 2003) and the patch-clamp technique (Carmichael, 2008) have been developed to date, the BERA method has advantages over these methods because of its ability to detect analytes in a specific, fast (less than 3 minutes) way, remarkably reproducible and economical. In addition, it is user-friendly, thus allowing the application of high throughput tests. Until now and in the case of nerve cells, the BERA method has been used to detect neuroactive toxic substances, such as pesticides (Momoeda et al., 2014; Lucks, 2003; Martelle et al., 2008).

In the present work, we present a novel approach for developing a functional assay for the in vitro interaction of N2a cells with DA using the BERA method. The new approach is characterized by significant speed (3 minutes), sensitivity (DA concentration as low as 1 nM) and reproducibility, while allowing precise monitoring of DA overflow pattern by cultured cells, along with cyclic voltametry. In addition to the interaction of N2a with DA, the effects of both eticlopride, a D2 antagonist on dopamine, and sodium ions were also evaluated.

Eticlopride is a high affinity and selectively substituted benzamide for dopamine D2 receptors, originally developed as a potential antipsychotic. Additionally, it was found to inhibit, at very low doses, the apomorphine-induced syndrome (Martelle et al., 2008). Similar to other substituted benzamides, eticlopride, at high doses, induces a weak form of catalepsy. In vitro, the compound was found to strongly inhibit dopamine D2 receptors.

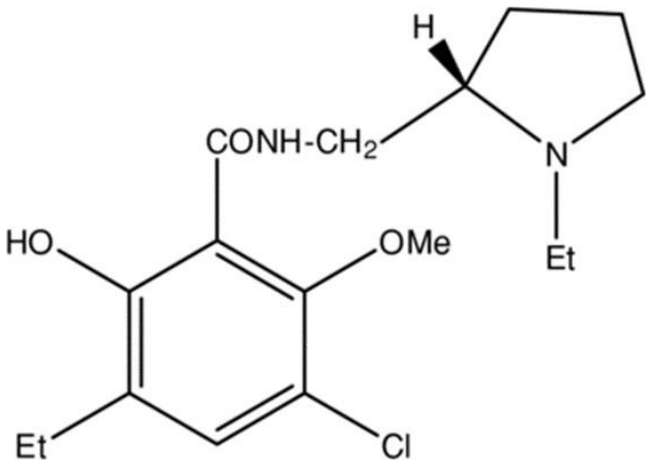


***Figure 12**. The simple chemical structure of Eticlopride.*

The approach developed in the present work was also used to evaluate the dopaminergic activity of chaste tree extracts (*Vitex agnus-castus*). Extracts of this plant have been shown to be helpful in treating various pathological conditions due to abnormal dopamine concentrations such as cyclical mastalgia (Carmichael, 2008), premenstrual mastodynia, cycle irregularities (Wuttke et al., 2003), mood alteration, headache and bloating (Liu et al., 2004). Extracts of *V. agnus-castus* have shown a considerable reduction in the above symptoms (Momoeda et al., 2014) and have been shown to act as DA agonists (Lucks, 2003).

Eventually, this approach was used to assess the activity of two antipsychotics, haloperidol, and olanzapine.

Haloperidol was discovered by Paul Janssen (Sneader, 2005) in 1958 and is considered to be one of the first-generation antipsychotic drugs. It is widely used to treat various conditions such as schizophrenia, Tourette syndrome, nausea and vomiting, and hallucinations in alcohol withdrawal (Plosker, 2012). To date, it is perhaps one of the safest and most effective antipsychotic drugs (Stevens, 2004).

Haloperidol has antagonistic action on D2 dopamine receptors and has a high affinity with a Ki value of 1.55 nM (Seeman & Tallerico, 1998). The drug preferably binds to the 5-HT2 receptors at high concentrations and to the D2 and α1 receptors at lower concentrations. As the positive effects on schizophrenia symptoms are associated with D2 receptor antagonism and negative effects with 5-HT2 receptor antagonism, this feature highlights the significant effect of haloperidol on hallucinations, and other psychosis (Schotte et al., 1993).

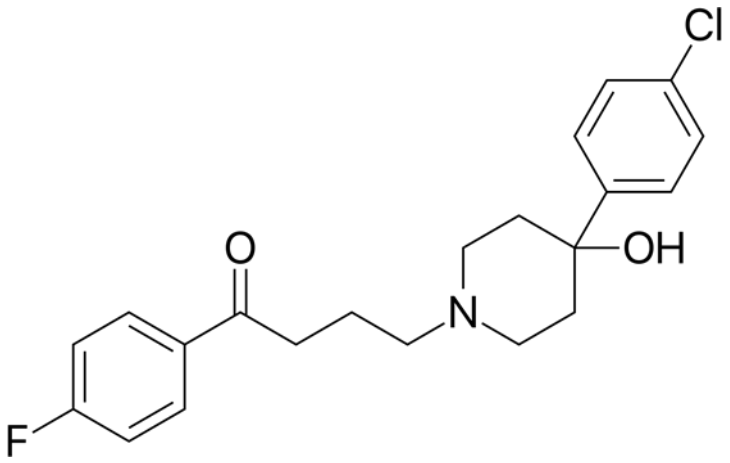


Figure 13. *Skeletal formula of haloperidol (original brand name Haldol)—a widely used typical butyrophenone antipsychotic. (By Fvasconcellos 19:33, 12 July 2007 (UTC) - PubChem, Public Domain, https://commons.wikimedia.org/w/index.php?curid=2393482).*

Olanzapine was discovered in 1971 and is considered as a second-generation antipsychotic drug. It was approved in the United States for use in 1996 (Taylor et al., 2015), and has since been used mainly for the treatment of schizophrenia and bipolar disorder. With regard to schizophrenia, the drug can be used both during the onset of the disease and for long-term maintenance. Studies have shown that olanzapine has fewer side effects compared to haloperidol, but caused a significant increase in cholesterol and triglyceride levels (Zhang et al., 2013). A review by Citrome et al. (2012) showed that compared to first-generation antipsychotics, only three of the second-generation antipsychotics (clozapine, olanzapine, and risperidone) were better. Olanzapine compared to Haloperidol have a lower affinity for D2 receptors with a Ki value of 3.00 nM (Seeman & Tallerico, 1998).

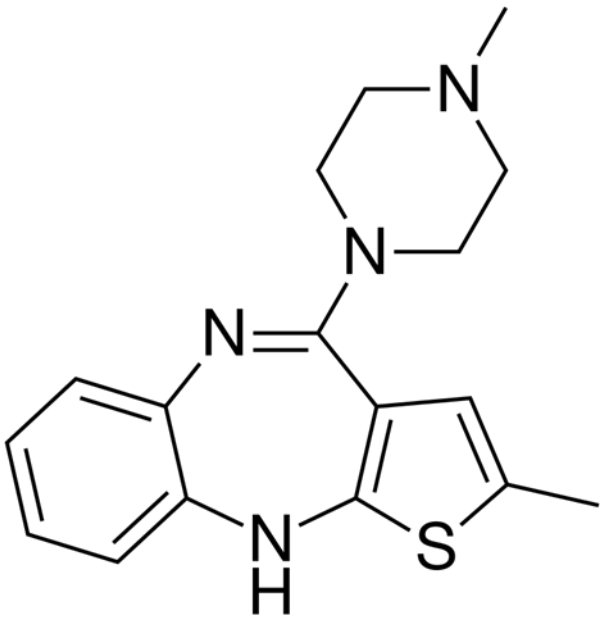


***Figure 14**. 2D structure of the antipsychotic drug olanzapine (original brand name Zyprexa)—a thienobenzodiazepine derivative. (By Harbin - https://pubchem.ncbi.nlm.nih.gov/compound/4585)*

2.3 Materials and methods

Chemicals

The chemicals: Eticlopride (5-chloro-3-ethyl-N-[[(2S)-1-ethyl-2-pyrrolidinyl]methyl]-2-hydroxy-6-methoxybenzamide), Choline chloride ((2-Hydroxyethyl)trimethylammonium chloride), Sodium chloride (NaCl) and Dopamine hydrochloride (2-(3,4- Dihydroxyphenyl) ethylamine hydrochloride) were purchased from Sigma-Aldrich (Taufkirchen, Germany).

Culture medium

Dulbecco's modified Eagle's medium (DMEM) in the presence or absence of Phenol Red, penicillin/streptomycin solution, L-glutamine solution and pyruvate acid solution (Biochrom AG) were used for the growth media of the cell lines. Fetal bovine serum (FBS) was obtained from Invitrogen (Massachusetts, USA) and the trypsin / EDTA solution from Biochrom AG (Berlin, Germany)

Propagation and Maintenance of Mouse N2a Cells

The cell line used in the present study was N2a cancerous neuroblasts (mouse brain neuroblastoma / *Mus musculus, Neuro-2a, ATCC® CCL-131 ™) and were originally provided from LGC Promochem (UK).*

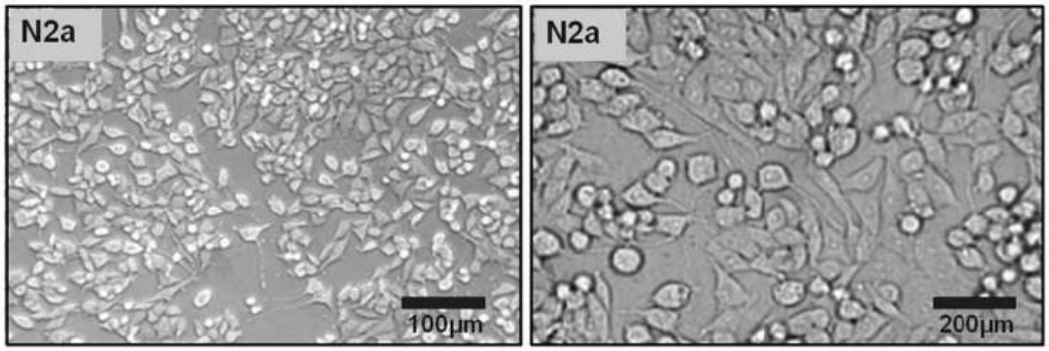


Figure 15. *Photomicrographs of N2a cells under light inverted microscopy (A. Krüss-MBL3200) under different magnifications.*

Cell lines were grown and maintained in monolayer cultures in 75 cm^2 culture flasks (OrFlask, Orange Scientific) in DMEM medium (20 mL) (Biochrom AG) which was enriched with 10% FBS (Invitrogen), 1% L-glutamine (Biochrom AG), 0,5% Pyruvate acid solution (Biochrom AG) and 1% antibiotic solution (Biochrom AG) in a suitable incubator (Heal Force® Model HF90) at 37 °C and in a steam-saturated atmosphere with a 5% CO_2 continuous flow.

Table 7. *Composition of DMEM*

Ingredients	***(mg/l)***	***Ingredients***	***(mg/l)***	***Ingredients***	***(mg/l)***
NaCl	*6400*	*L- methionine*	*30*	*Myo-inositol*	*7.2*
KCl	*400*	*L-phenylalanine*	*66*	*Nicotinamide*	*4*
$CaCl_2$	*200*	*L-threonine*	*95*	*L-arginine·HCl*	*84*
$MgSO_4 \cdot 7H_2O$	*200*	*L-tryptophane*	*16*	*L-cystine*	*48*
NaH_2PO_4	*124*	*L-tyrosine*	*72*	*L-glutamine*	*580*

D-glucose	*1000*	*L-valine*	*94*	*L-histidine·HCl·H2O*	*42*
$Fe(NO3)_3 \cdot 9H_2O$	*0.1*	*L-Glycine*	*30*	*L-isoleucine*	*105*
Na-pyruvate	*110*	*L-serine*	*42*	*L-leucine*	*105*
Phenol red	*15*	*Cholin chloride*	*4*	*L-lysine·HCl*	*146*
$NaHCO_3$	*3700*	*Folic acid*	*4*	*D-Ca-pantothenate*	*4*
Riboflavin	*0.4*	*Thiamine·HCl*	*4*	*Pyridoxal·HCl*	*4*

Freeze and thaw cell lines

Preserving cells for a long time is achieved by storing at -80 °C. Freezing is done gradually so that the cells are not shocked by the temperature difference. After detaching and collecting the cells from the flask, the number is measured and the number is adjusted by appropriate reduction. Then they are transferred to appropriate cryovials with heat-inactivated FBS (56 °C / 30min) in the presence of 10% Dimethyl sulfoxide (DMSO). DMSO inhibits the formation of water crystals inside the cells during cooling. With the addition of DMSO, the freezing procedure is immediately initiated to avoid the toxicity of the cells to ambient temperature. The freezing is done gradually, first, the cryovials are added on ice (4 °C) where they remain for 1h. Then, they are placed at -20 °C for another 1h and finally transferred to -80 °C where they can remain for a long time and after thawing to re-cultivate.

The process of defrosting cell lines is performed under aseptic conditions and as quickly as possible in order for the cells not to be exposed to DMSO, which is likely to act toxic. After removing them from -80 °C, the cryovials are placed directly in a 37 °C water bath until they are thawed. Then the solution of the cells obtained after thawing is diluted in DMEM / 10% FBS medium and added into a 75 cm^2 flask where it is spread throughout its surface for better and uniform growth of the cells. The volume of the solution is 20 ml. Finally, the flask is placed in the incubation chamber (37 ° C, 5% CO_2) where the incubation of the cells is carried out.

Subculturing Adherent Cells

The cells are cultured until their monolayer covers 90% -100% of the surface of the

flask. This condition is controlled by direct observation of the flask using a reversed microscope. The passage of the cells is done by detaching the cells with trypsin / EDTA solution. Incubation in trypsin lasts 5 min at 37 °C followed by resuspension of the detached cells in 10% FBS enriched nutrient medium. The cells are then centrifuged to remove trypsin, resuspended in a complete nutrient medium and transferred to new culture flasks. The passage of cells is carried out under aseptic conditions in a laminar airflow cabinet.

Cell counting - Determination of viability

Cell viability is defined as the ratio of the number of live cells to the total number of cells, as measured on a Neubauer hemocytometer, after adding a trypan blue dye solution. Cells absorbing trypan blue dye are considered as dead.

Into the cell suspension is added a solution of 0.4% w / v trypan blue dye (in PBS) in a ratio of nine to one so that the final concentration of the dye is 0.04% w / v. Incubate for 5 minutes at room temperature. The cells are placed on the hemocytometer and counted. From the number of cells dyed blue due to dye entry, the percentage of viability is calculated according to the ratio:

Percentage of viability% = 100 x [1 - number of dead cells (blue) / number of cells measured]

The total number of cells in the suspension is calculated by:

Cell count / mL = number of cells x 10^4 x dilution

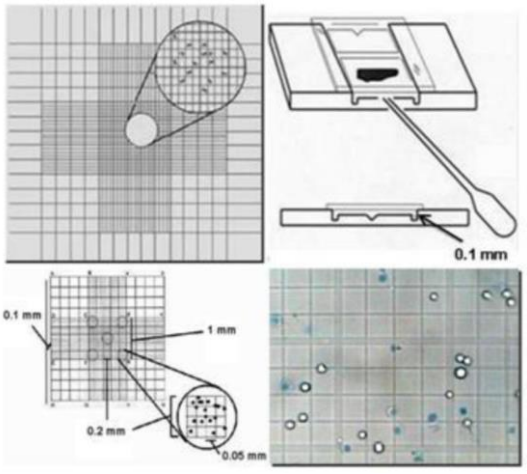


***Figure 16**. Hemocytometer - Neubauer plate. The volume of the cell suspension covering the measuring surface is 0.1mm3 or 1 x 10-4ml. Dead cells are stained blue.*

Cell membrane potential measurements: biosensor set-up

The potential of the membrane is the difference in electrical potential between the inside and outside of a biological cell. The cell membrane consists of a lipid bilayer with ion pumps embedded therein, which push active ions through the membrane and establish concentration gradients across the membrane, and ion channels that allow the ions to move through the membrane.

When the nerve cells are in a state of rest, the inside of their membrane is more negative than the outside (typically about -70 millivolts). Opening and closing the ion channels can cause deviation from the resting potential. This situation is called depolarization when the internal voltage becomes less negative and hyperpolarization when the internal voltage becomes more negative.

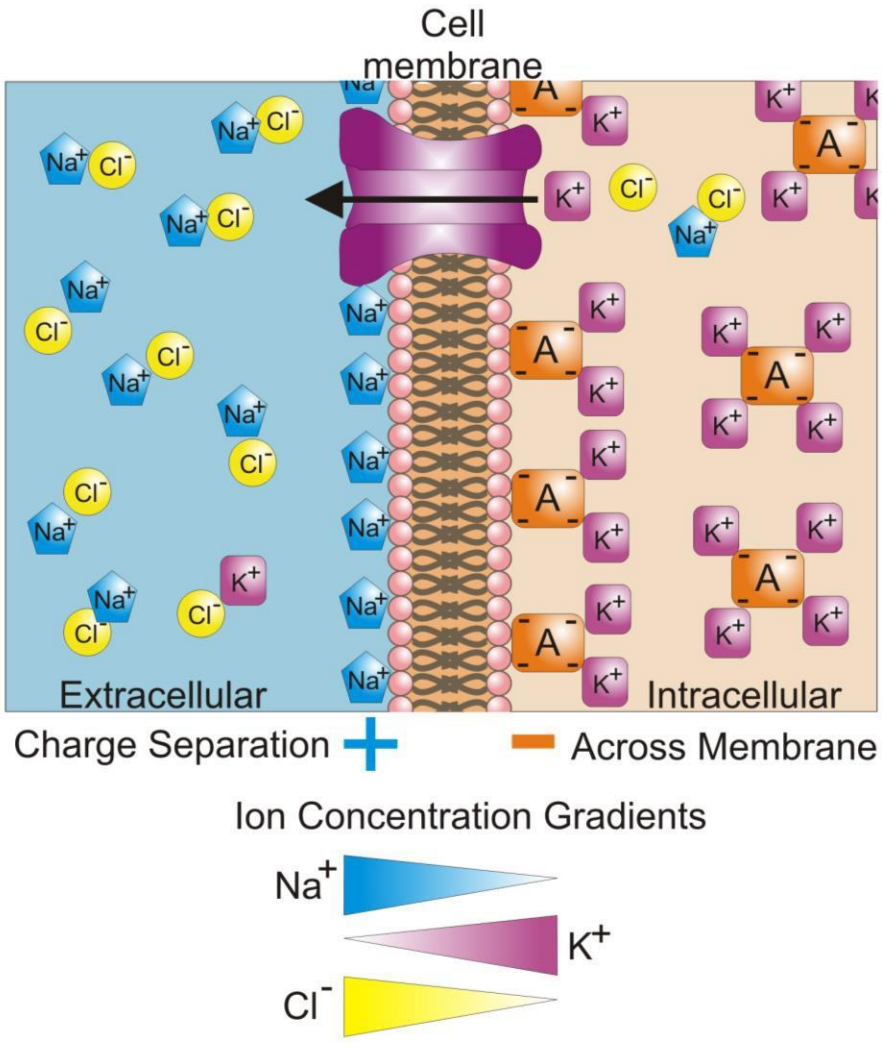


Figure 17. *Differences in ion concentrations on opposite sides of a cell membrane lead to a voltage difference called membrane potential. (Figure adapted from By Synaptidude, CC BY 3.0, https://commons.wikimedia.org/w/index.php?curid=21460910)*

Cell membrane potential changes were measured using a personalized, multi-channel potentiometer (Uniscan, Buxton, UK), which allowed up to eight simultaneous measurements from eight electrodes printed on a disposable sensor tape (DropSens, Asturias, Spain) (Figure 18A). The reference electrode material was Ag / AgCl.

Prior to the start of the measurements, the cultured cells were isolated and concentrated by centrifugation (2 min, 1200 rpm, 25 °C). The cells in suspension, with the aid of a multi-channel automatic pipette, were first added to the top of each of the eight electrodes located in each disposable sensor strip (45 µL ≈ 50 x 10^3 cells) (Figure 18B) and then 5 µl of sample (DA solution) were added onto the cells (Figure 18C).

Immediately after the addition of different samples, a cell response was recorded, which was presented as a time series of potentiometric measurements (in Volts) (Figure 18D). For the sake of proper comparison of measurements between different concentrations, during the analysis phase, the potentiometric response of DA recorded without the presence of cells was subtracted from the observed cell response for each experiment. Each measurement was 3 minutes with a sampling rate of 2 Hz.

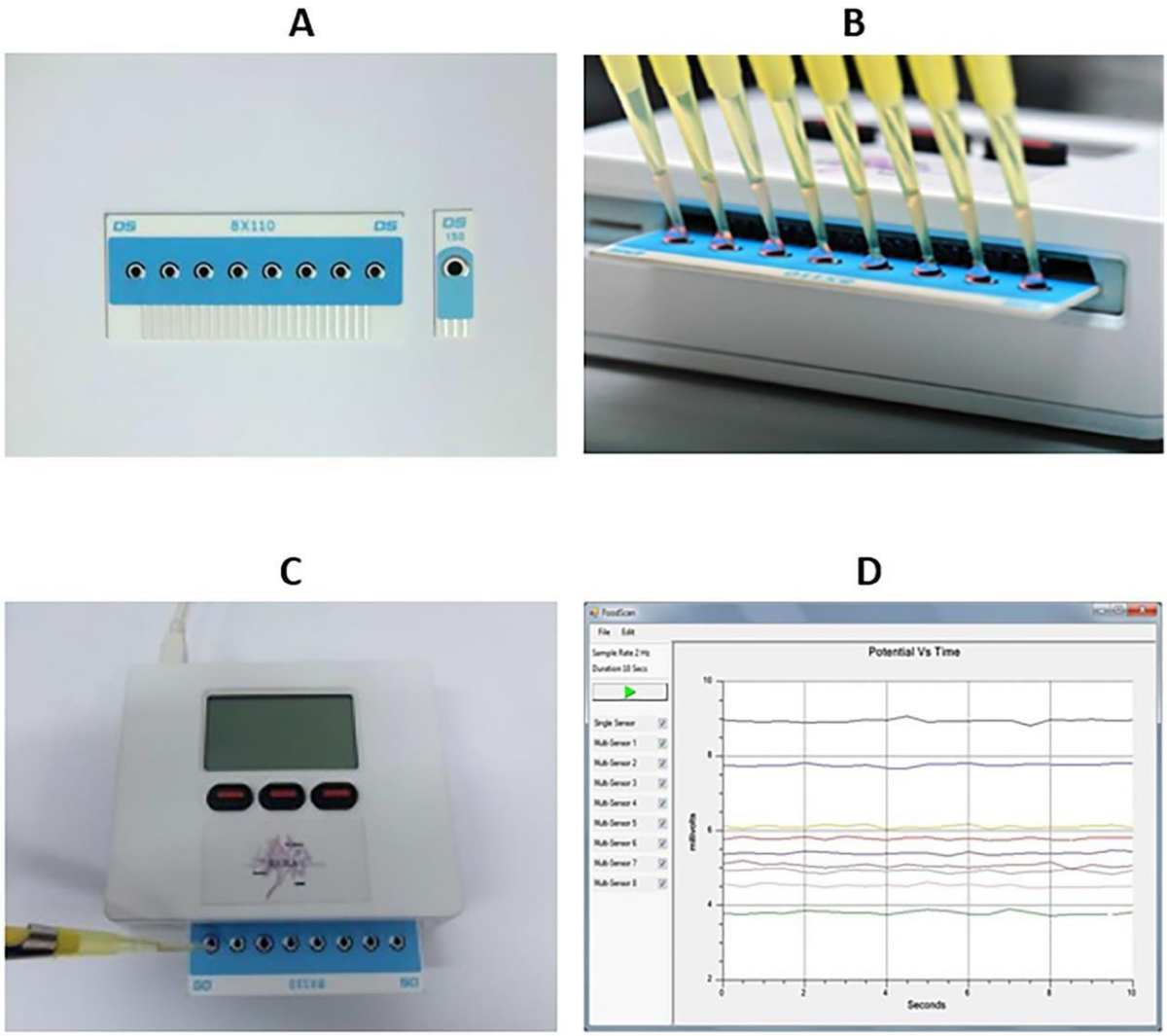


Figure 18. (A) *Single and eight-channel disposable sensors,* ***(B)*** *Adding a cell suspension at the top of each of the eight electrodes,* ***(C)*** *Adding a sample (DA solution) to the cell suspension,* ***(D)*** *Real-time measurements using a specific software.*

The response of cells to different concentrations of eticlopride antagonist was examined in order to study the effect of this antagonist (Martelle et al., 2008). For the purpose of comparison of the results, both DA and eticlopride solutions were prepared on the same day using double distilled water. At the same time, since studies (Grigoriadis & Seeman, 1985) have shown that sodium chloride was able to completely convert D2 receptors from a high affinity for DA to a low-affinity state, the cells were first incubated with sodium chloride at a concentration of 120 mM and then transferred to a biosensor for treatment with DA. To exclude the effect of chloride ions, cells were incubated with

120 mM choline chloride prior to transfer to the biosensor.

Estimation of DA overflow

Utilizing the cyclic voltammetry and setting the sweep rate to 100 mVs^{-1} and scanning potentials between -1.0 V and +1.0 V, we checked the overflow pattern derived from the cultured N2α cells. N2α cells at a density of 10^6 were initially incubated for three minutes in eppendorf tubes, at 50 μl of different concentrations of DA that had previously been made in the DMEM medium. Subsequently and after the incubation, eppendorfs were centrifuged and the supernatant collected was assessed for DA determination. After analyzing each voltammogram, and in particular the oxidation range of the substance, it was found that the magnitude of the change in current (in μA) corresponded to +0.37 V, which was then used to indirectly determine the DA concentration. The biosensor was calibrated at various DA concentrations ranging from 0 μM to 1000 μM. Each calibration test was performed three minutes after the fresh DA solutions were prepared, so the calibration data could very well be compared to the results obtained from the release-uptake balance measurements.

Evaluation of the dopaminergic activity of chaste tree extracts (Vitex agnus-castus)

To extract DA from *V. agnus-castus*, initially one gram of immature fruit was added to 10 mL of 70% methanol and homogenized, with the homogenate then inserted for 20 minutes in an ultrasonic bath. Subsequently, the sample was filtered using Whatman filter and the extract was diluted in water to give final concentrations of 1% and 10% (v / v). Dopaminergic activity was assessed by measuring the changes in cell membrane potential after the addition of 5 μl of sample to N2a cells that had or had not previously been treated with 5 μl of eticlopride solution.

Estimation of the effect of antipsychotics haloperidol and olanzapine

For investigating the effects of two well-known antipsychotics which act as D2-like receptor antagonists with a lower affinity than eticlopride, cells were treated with different haloperidol and olanzapine concentrations with the same concentration range to DA. For the purpose of comparison of the results, DA, haloperidol, and olanzapine solutions were

prepared on the same day using double distilled water.

Experimental design

The experimental design was followed by a completely randomized design with three technical replicates. Each experiment was repeated 5 times in total on different days and the response of each sample x biorecognition element combination was measured eight times. All the results are expressed as the mean ± SEM. Differences between the means were tested for statistical significance using an analysis of variance.

2.4 Results and Discussion

Response of N2a cells to DA concentrations

Two different patterns, depending on the DA concentration range, were demonstrated by the biosensor response, after analyzing the potentiometric measurements: (a) an increase in the cell membrane potential proportional to the increase in DA concentration between the concentrations 0-100 nM, indicating membrane depolarization (Figure 20A) and (b) a decrease in membrane potential, inversely proportional to the increase in DA concentration, between 1-1000 μM concentrations, indicating membrane hyperpolarization (Figure 20B). Assay of dopamine itself (without cells) produced a similar response irrespective of its concentration as shown in Figure 19.

Previous studies (Grace & Bunney, 1984; Missale et al., 1998) have shown that different DA receptors have different DA affinity. For example, D1-like receptors have the ability to induce depolarization of the cell membrane because they stimulate cAMP accumulation while D2-like receptors induce hyperpolarization of the cell membrane as they inhibit cAMP accumulation. Consequently, the observed results reveal a preference for DA in specific receptors depending on the concentration of DA: (a) at low concentrations D1 receptors (nanomolar range) are activated and (b) at high concentrations D2 receptors are activated (micromolar range) (Furman et al., 2009). The above results are in agreement with the findings of Trantham-Davidson et al. (2004) who showed that D1-like receptors in the frontal cortex are activated by low concentrations (<500 nM) while D2-like receptors by high concentrations (> 1μM).

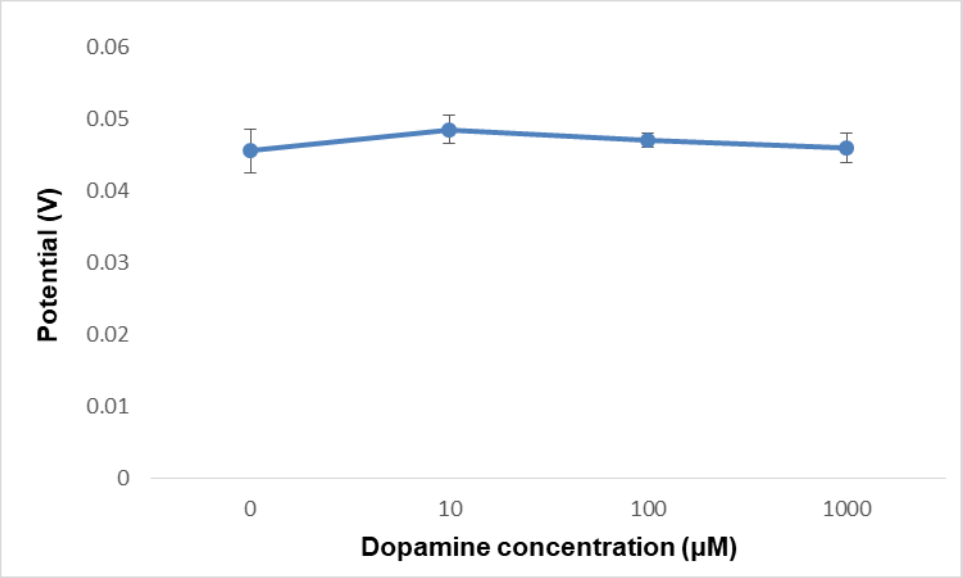


Figure 19. *Biosensor response to different concentrations of Dopamine without the presence of cells. Assay of dopamine itself (without cells) produced a similar response irrespective of its concentration revealing no response of the device to the different dopamine concentrations.*

In addition, the new biosensor approach was used to study cell membrane potential changes induced by the presence of eticlopride, which has been described as a selective D2-like receptor inhibitor. As expected because of the ability of eticlopride to block D2-like receptors, depolarization of the cell membrane was observed by shifting responses to positive values (Figure 20B). In contrast, incubation of cells with 120 mM NaCl resulted in attenuation of cell membrane hyperpolarization observed with a shift of responses to positive values (Figure 20B).

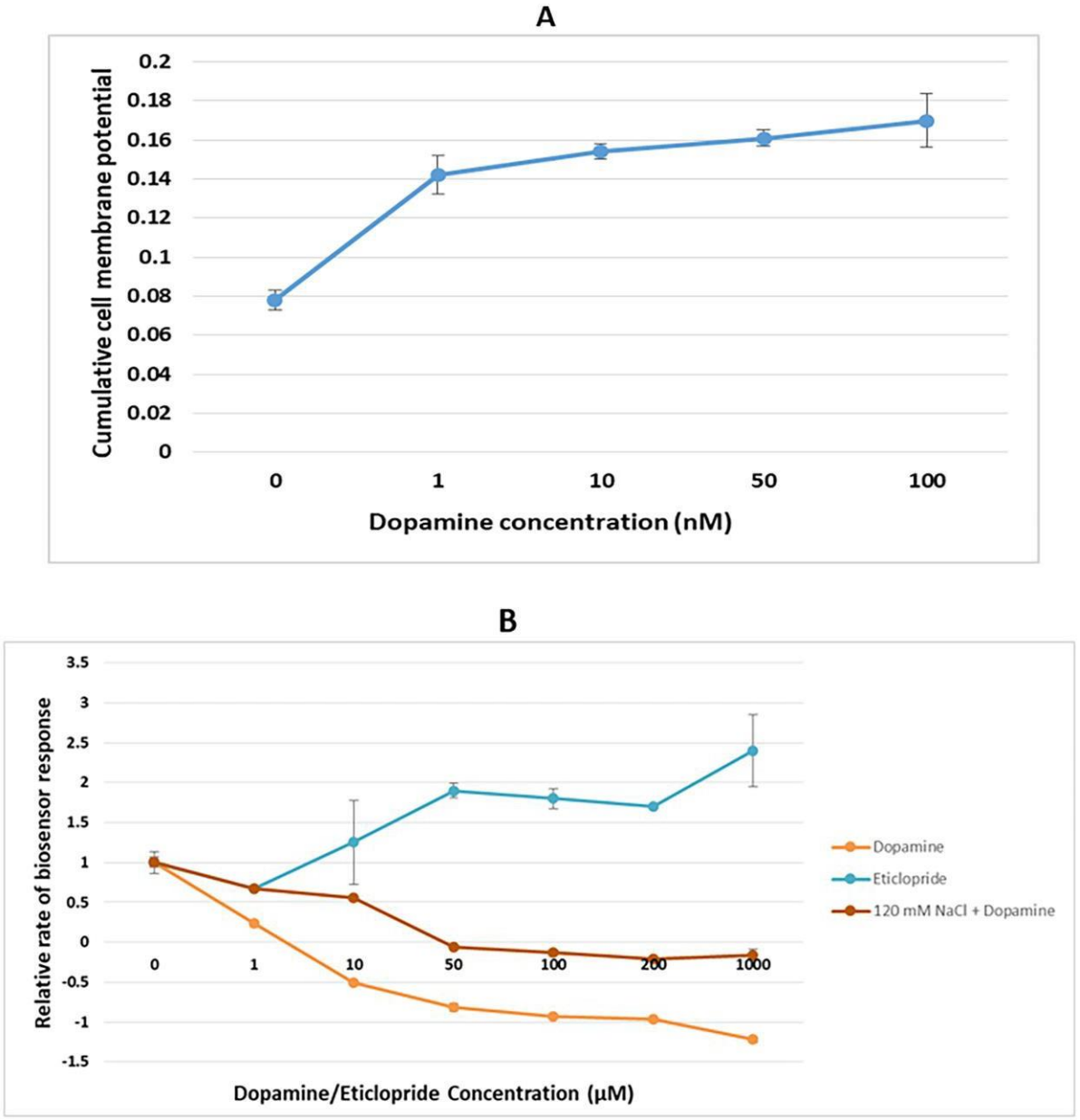


Figure 20. (A) *Increase in the cell membrane potential proportional to the increase in DA concentration between the concentrations 0-100 nM, indicating membrane depolarization.* ***(B)*** *Biosensor responses to different treatments. Results are presented in relation to control (no treatment). Decrease in membrane potential, inversely proportional to the increase in DA concentration, between 1-1000 µM concentrations, indicating membrane hyperpolarization. Cell response to DA is shown after removing the effect of DA alone (cell-free measurement) on the biosensor system. In contrast, eticlopride treatment caused a completely opposite, concentration-dependent, shift to positive values indicating membrane depolarization. Incubation of N2α cells in*

120 mM NaCl also caused a shift to positive values at all DA concentrations, relative to DA treatment alone, thereby revealing a diminished DA receptor affinity. Each data point represents the average of three individual experiments, each with eight measurements per sample.

The observed results were expected as previous studies have shown that the affinity of D1-like and D2-like receptors to DA and its agonists decreases in the presence of sodium ions (Grigoriadis & Seeman, 1985). At this point, it is worth noting that the chloride ions did not have a similar effect as the sodium ions, as when the cells were incubated with choline chloride the response in the presence of DA was relatively similar to the response of the cells in the control sample (r^2 = 0.98861) (Figure 21).

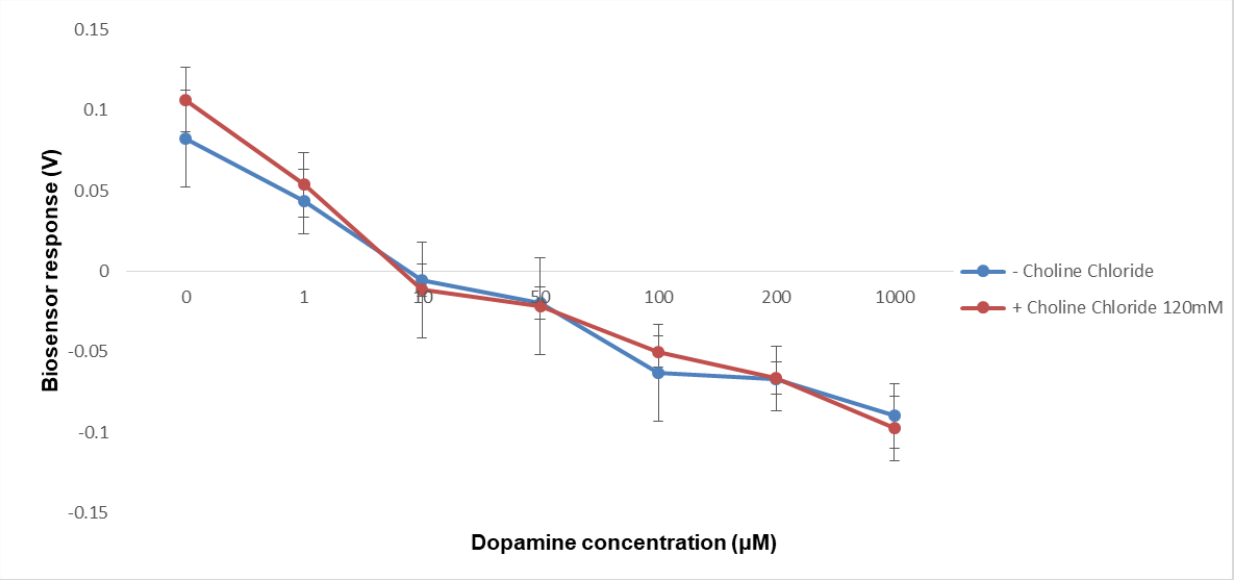


Figure 21. *Biosensor responses to non-identical treats. Membrane hyperpolarization of N2α cells in the presence of increasing DA concentrations ranging in the micromolar range (1-1000 μM), as observed by potentiometric assay of cell bioelectric potential. An almost similar response to N2a cells incubated with choline chloride (r^2=0,98861).*

The results observed in every experiment were highly reproducible, with a mean standard error of 4.4%.

DA overflow by N2a cells

The cyclic voltametric results of the DA test showed an overflow pattern of DA, which was concentration dependent. The results revealed an almost linearly pattern (r^2 = 0.902) after the application of exogenous DA in the range of 1-1000 μM (Figure 22). In this way, the DA overflow pattern was inversely correlated with (r^2 = -0.804) with the noticed changes in cell membrane potential as recorded by the new assay.

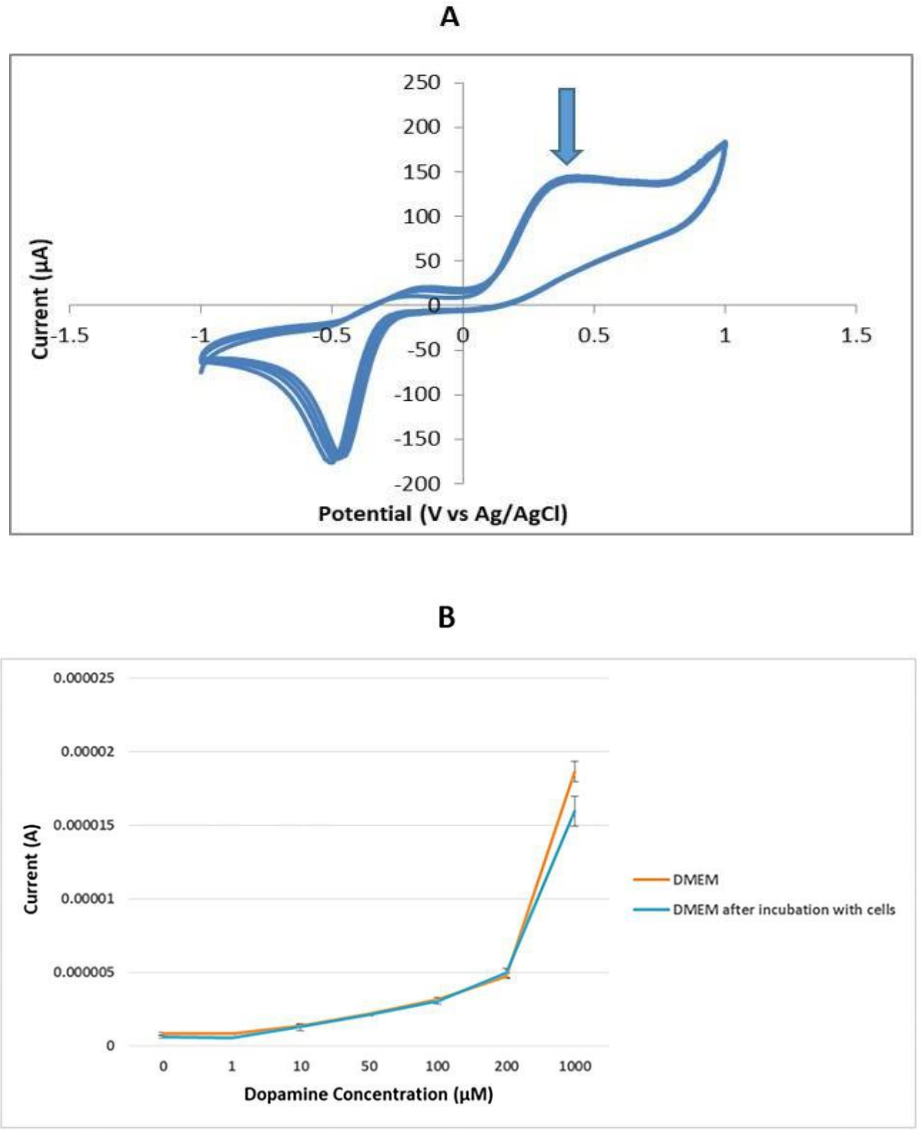


Figure 22. (A) *A set of five consecutive dopamine cyclic voltammograms (at 1000 μM). The oxidative peak at 0.37 V is indicated by the arrow.* ***(B)*** *A dopamine calibration curve (orange) and changes in dopamine release-uptake equilibrium from cells to increasing dopamine concentrations (blue). The data represent the average of three individual experiments, each with eight measurements per sample.*

Evaluation of dopaminergic activity of V. agnus-castus extracts.

After the assessment of dopaminergic activity of the extracts that came from chaste tree (*V. agnus-castus*) with the new assay, the results (Figure 23) revealed that, when 1% extract concentration was applied, the samples did not induce any considerable cellular response as calculated by the differences between control cells (blue column) and cells that treated with eticlopride (orange column).

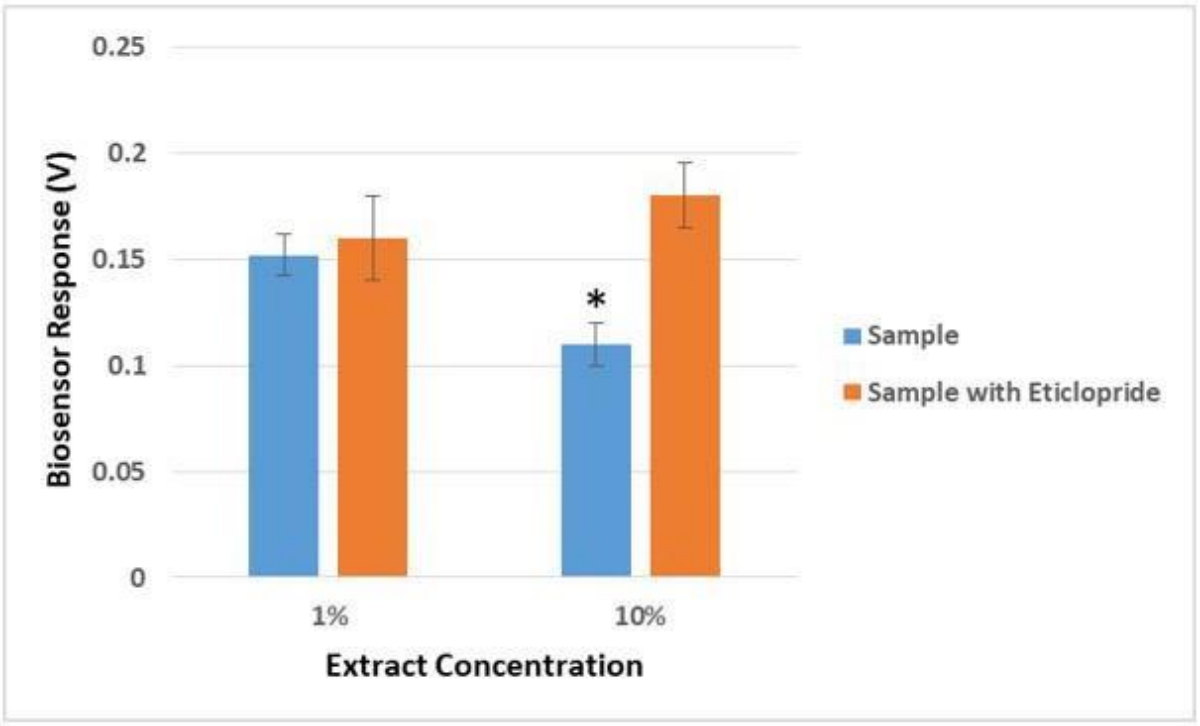


***Figure 23**. Measurement of cell membrane potential change of N2a cells in fruit extracts of V.agnus-castus for evaluation of their dopaminergic activity. Before applying the sample, the cells were treated (orange column) or not (blue column) with eticlopride. Thus, any dopaminergic activity of the samples could be observed in control cells (not treated with eticlopride). Data correspond to the mean ± SEM of three different experiments * p <0.05.).*

The non-significant differences between the cells at the lower concentration of the extract are most likely due to the fact that the concentration of agonists or DA in the sample was very low. In contrast, application of *V. agnus-castus* extracts at a concentration of 10% (i.e. 10-fold) elicited a relatively significant ($p < 0.05$) lower response

to the control cells compared to the cells treated with eticlopride, thus revealing hyperpolarization of the cell membrane due to the presence of dopamine-agonist in the extract. As expected, after the addition of the D2-dopamine receptor antagonist prior to the addition of 10% *V. agnus-castus* extract, no significant response was observed.

Biosensor responses to antipsychotics haloperidol and olanzapine

The results of the biosensor analysis treated with eticlopride, a highly selective D2 receptor inhibitor, revealed a concentration-dependent shift of bioelectric potential to positive values indicating membrane depolarization. In order to investigate further that response of the biosensor-based functional assay, cells were treated with antipsychotics haloperidol and olanzapine which act as a D2-receptor antagonist with lower affinity than eticlopride (Figure 24).

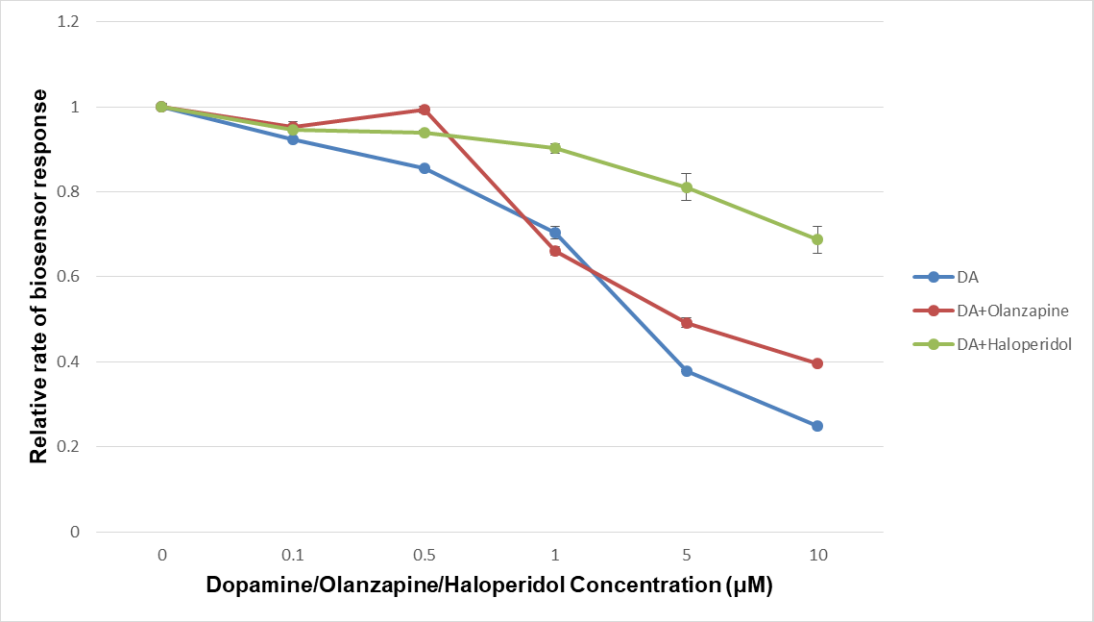


Figure 24. *Biosensor responses to different treatments. Results are presented in relation to control (no treatment). Membrane hyperpolarization of N2a cells in response to increasing DA concentrations, as measured by potentiometric analysis of cell bioelectric potential. Treatment with olanzapine and haloperidol caused a shift towards positive values, relative to treatment with DA only, revealing a weakening of D2-receptor affinity.*

Olanzapine and haloperidol cause a shift in the bioelectric potential, depending on

the concentration of the substance, to more positive values, indicating a significant attenuation of the DA-related hyperpolarization effect. The observed results are comparable with the previous finding, of our biosensor-based functional assay, showing a concentration-dependent membrane depolarization when cell treated with D2-receptor antagonist eticlopride. Although, this time the shift toward positive values was smallest compare to eticlopride because haloperidol and olanzapine have almost two times and four times, respectively, less affinity on D2-receptors relative to eticlopride. On the other hand, haloperidol with a higher affinity on D2-receptors than olanzapine causes a higher shift towards positive values (Seeman & Tallerico, 1998).

2.5 Conclusions

In the present study, a biosensor-based functional assay method was developed, where the results showed that it could be used to develop a fast, highly sensitive high-performance screening system for DA agonists and/or antagonists, to evaluate their effect on whole cellular responses in vitro. Further, as demonstrated in the present study, the assay can be easily combined with several advanced biosensor (mainly electrochemical) techniques to rapidly determine DA concentration and its overflow rate after administration of agonist or antagonist molecules (Jackowska & Krysinski, 2013), thereby significantly increasing the number of information that can be retrieved from a single in vitro experiment. As has also been shown, in the case of immature *V. agnus-castus* fruit extracts, this bioelectric approach could very well be a useful tool for rapidly assessing the dopaminergic potency of natural bioactive drugs to be used in clinical trials or ethnobotanical studies.

Chapter 3

Non-chemical, distant cellular interactions between neuronal cells

Part of this chapter has been published in the form of original research article with the following data: Apostolou T, Kintzios S, (2018) Evidence of near-instantaneous distant, non-chemical communication between neuronal (human SK-N-SH neuroblastoma) cells by using a novel bioelectric biosensor. Journal of Consciousness Studies 25: 62-74(13).

3.1 Abstract

The mechanism of cell-to-cell communication and the coordinated cellular responses they present is something that has been of great concern to the scientific community in recent years. Advanced, innovative reseacrh focuses on non-chemical, distant cellular interactions (NCDCI) that are likely to be responsible for this communication. Recent experiments have suggested the field theory of conscious electromagnetic information (CEMI) as a possible explanation for this cell-to-cell communication. In the present work, using a bioelectric biosensor, we observed the emergence of distant communication between neuroblastoma cells providing supporting evidence for this theory. This observation was made simultaneously with the observations of changes in the membrane potential of human SK-N-SH neuroblastoma cells that were physically separated from each other. The cells were divided into two groups naturally separated. In one group we had the "inducer" cells that were stimulated with dopamine, and in the other group, we had the "detector" cells which showed a synchronized response to the "inducer" cells, with the amplitude of the response decreases as the distance increases. In order to investigate the nature of the mechanisms that cause the observed distant cell interactions, cell cultures were separated with barriers, which is non-transparent in a certain frequency or treated with vinblastine, a vinca alkaloid, which binds tubulin, thereby inhibiting the assembly of microtubules. The mechanism responsible for cell-to-cell communication is discussed in accordance with the observed effects of coordinated changes in membrane potential.

3.2 Introduction

The first experiments related to the principle of non-chemical, non-direct electrical communication between biological systems were carried out by Gurvitch in 1920. Gurvitch had shown that onion root cells that were chemically isolated from each other, when located close to actively dividing cells, showed increases in the number of mitoses. Unfortunately, this study, along with other similar studies, was never published in English and, therefore, did not attract much attention in the Western world.

During the last two decades, several experiments have been carried out that have demonstrated the existence of non-chemical communication between cells naturally separated from each other (Wainwright, 1998; Nikolaev, 2000; Trushin, 2003, 2004; Fels, 2009; Cifra et al., 2011). Various terms have been proposed to describe these phenomena with the predominant being non-chemical, non-electrical (NCNE) signaling, and non-chemical, distant cellular interactions (NCDCI) (Wainhright, 1998; 2014). In 2007 Farhadi and his team placed Caco-2 cell cultures in separate containers that were in contact with each other. Then they observed that with the application of H_2O_2 ssolutions to the first container which was termed " inducer ", a similar effect was observed in the second container (" detector ") which in this case was not exposed to H_2O_2. The analysis of the results showed a synchronized response between the two containers (" inducer " - " detector ") which translated to a decrease in total protein content, damage to the cytoskeleton and increased activation of nuclear NFκB. In a later work, Chaban et al. (2013) observed a synchronous response to dorsal root ganglia (DRG) neurons when they were placed, in close distance, with physically detached apoptotic DRGs or human neuroblastoma (SH-SY5Y) cells. This response resulted in a significant alteration of ATP- and capsaicin-mediated $[Ca^{2+}]i$ influx. Hashemibeni and his team (2014) observed the presence of NCDCI among stem cells derived from adipose tissue, expressed as alterations in cell proliferation possibly due to the proximity of inducer cells (fibroblast growth factor-treated cells). In the past years, Fels (2016) demonstrated the effect of naturally occurring autotrophic single-cell cultures of *Euglena viridis* on the rate of proliferation of the heterotrophic monocyte *Paramecium caudatum* and the multicellular heterotrophic *Rotatoria sp*.

So far, several mechanisms have been proposed by the scientific community in their attempt to explain the communication between distant cells. Most observations lead to electromagnetic radiation as the cause of this communication (Cifra et al., 2011; Reguera, 2011), and more specifically to the emission of biophoton with a wavelength of 200 to 800 nm, which results in the oscillation of the microtubules and/or the production of free radicals (Prasad et al., 2014; Tang and Dai, 2014; Pospíšil et al., 2014; Craddock et al., 2012). Specifically, molecular simulation experiments have shown a relationship between reactive oxygen species (ROS) and the phosphorylation state of microtubule proteins such as tau, especially with respect to UV-mediated information (Craddock et al., 2014, Kurian et al., 2017). At this point, it should be noted that evidence of the existence of electromagnetic cell-to-cell communication remains a challenge, mainly due to the dubious ability of cells to process electromagnetically mediated information, as well as the very small effect of such radiation on biological systems (Kučera and Cifra, 2013). The existence of distance cell-to-cell communication due to volatile substances have also been suggested but has not been demonstrated (Hashemibeni et al., 2014). In addition, another possible explanation for this particular communication is the presence of sound waves as demonstrated in bacterial experiments (Matsuhashi et al., 1998).

Verifying the electromagnetic interaction between nerve cells would significantly increase the evidence for a neuro-electromagnetic field capable of interacting with the brain to form a unified field of consciousness (Jones, 2017).

In the present work, we investigated the existence of NCDCI among human SK-N-SH neuroblastoma cells using a new biosensor that allowed real-time monitoring of the bioelectric properties of physically separated cells. We show that changes in membrane potential due to the addition of dopamine to inducer cells can be detected almost immediately by neighbouring cells (detector cells) which in this case are not exposed to dopamine and are naturally isolated from the inducer cells, to exclude chemical exchange phenomena. In the absence of dopamine, responses among distantly located cells were also observed and independent of the relative distance between them. In order to investigate the nature of mechanisms underlying the observed distant cellular interactions, cells cultures were separated with barriers, which is non-transparent in a certain frequency or treated with vinblastine, a *vinca* alkaloid, which binds tubulin, thereby inhibiting the assembly of microtubules.

The cytoskeleton, which consists of microtubules, actin filaments and intermediate filaments, is considered an organizational structure of the eukaryotic cell. Microtubules are of particular interest since they exhibit various characteristics that distinguish them from other subunits of the cytoskeleton. The building units of microtubules are the tubulin heterodimers consisting of α- and β-tubulin. (Figure 25). The protein subunits of tubulin, which are responsible for the formation of microtubules, form an architecture of chromophores, for example, aromatic amino acids, which include tryptophan. These formations exhibit some geometric and dipolar properties, similar to those of aromatic and at the same time similar properties to those found in photosynthetic units. These findings suggest that tubulin may be able to support coherent energy transfer. Tubulin agglomerated in microtubule geometries can support such energy transfer, which may be important for the biological signaling and communication required for living processes.

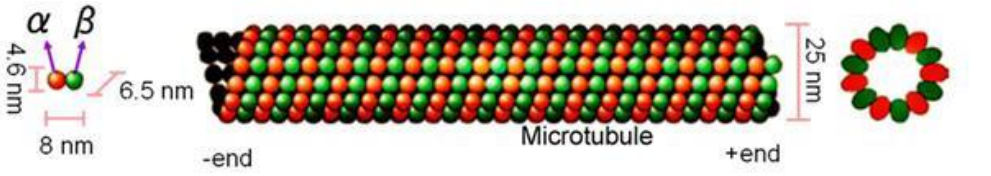


***Figure 25**. Structure of the microtubule (Sahuetal S. 2013)*

The use of Vinca alkaloids in the clinic in recent years has been a great success and is responsible for many chemotherapy success stories. Vinblastine and vincristine, which are natural members of this family, were isolated from the leaves of the plant *Catharanthus roseus*. Vinca alkaloids mainly target tubulin and microtubules, thereby depolymerizing the microtubules and destroying mitotic spindles, leaving the cancer cells blocked in the phase of mitosis with condensed chromosomes. Vinblastine has the ability to bind to the β-subunit of tubulin and therefore directly to the microtubules. *In vitro*, vinblastine binds to tubulin, with very high affinity, at the ends of the microtubules (Figure 26).

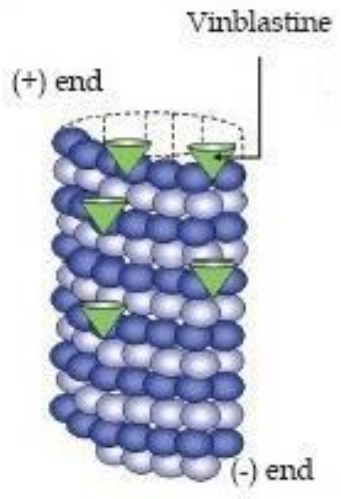


Figure 26. *Inhibitory patterns of microtubules by Vinblastine (Adapted from https://blogs.shu.edu/cancer/2016/02/17/halaven-shows-survival-advantage-in-liposarcoma-fda-approved/)*

3.3 Materials and Methods

Chemicals

Minimum Essential Medium (MEM) in the presence or absence of Phenol Red, penicillin/streptomycin solution, L-glutamine solution and pyruvate acid solution (Biochrom AG) were used for the growth media of the cell lines. Fetal bovine serum (FBS) was obtained from Invitrogen (Massachusetts, USA) and the trypsin / EDTA solution from Biochrom AG (Berlin, Germany). A monoclonal antibody against α-tubulin, phalloidin Atto 438, vinblastine, paraformaldehyde, BSA, Triton X-100 and the nuclear dye 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI) were purchased from Sigma-Aldrich (Germany).

Propagation and Maintenance of Human neuroblastoma SK-N-SH

The cell line used in the present study was human neuroblastoma SK-N-SH (ATCC HTB-11) and were originally provided from LGC Promochem (UK). The propagation and maintenance of the cells were the same as described in Chapter 2.

Biosensor set-up

Using a multi-channel potentiometer (Uniscan, Buxton, UK) specifically designed for this application, it was possible to measure changes in cell membrane potential, always in accordance with the Bioelectric Recognition Assay principle (Kintzios *et al*., 2001; Ferentinos *et al*., 2013). The system has the ability to show in real-time the measurements of changes in the electrical properties of nerve cells in the presence of various agonists and/or antagonists neurotransmitters (Apostolou *et al*., 2017). Allows taking from one to eight contemporaneous measurements from the corresponding positions (Positions 1-8), where each position comprises a pair of screen-printed electrodes (working electrode: carbon, reference: Ag / AgCl) in disposable sensor strip (DropSens, Asturias,) (Figure 27A). In the context of this experiment, dopamine-treated SK-N-SH cells ("inducer cells") were always placed in position 1, whereasuntreated SK-N-SH cells ("detector cells"), at positions 2, 3 or 4 (Figure 27B). With this experimental set-up, the effect of physical distance between cells treated with or without dopamine on the NCDCI signaling region could be observed.

Prior to each assay, cells were isolated from the culture and concentrated by centrifugation (2 min, 1200 rpm, 25°C). Before starting the assay, cells were placed at different positions on the electrode (45 μL ≈ 50 x 10^3 cells) by means of an automatic pipette. Then, 5 μl of a neurotransmitter solution (100 μM dopamine) was added (Figure 27A).

Instantly after dopamine added to the inducer cells, changes in the membrane potential of the inducer and the detector cells were recorded. The length of measurement was 3 minutes and were recorded at a 2 Hz sampling rate.

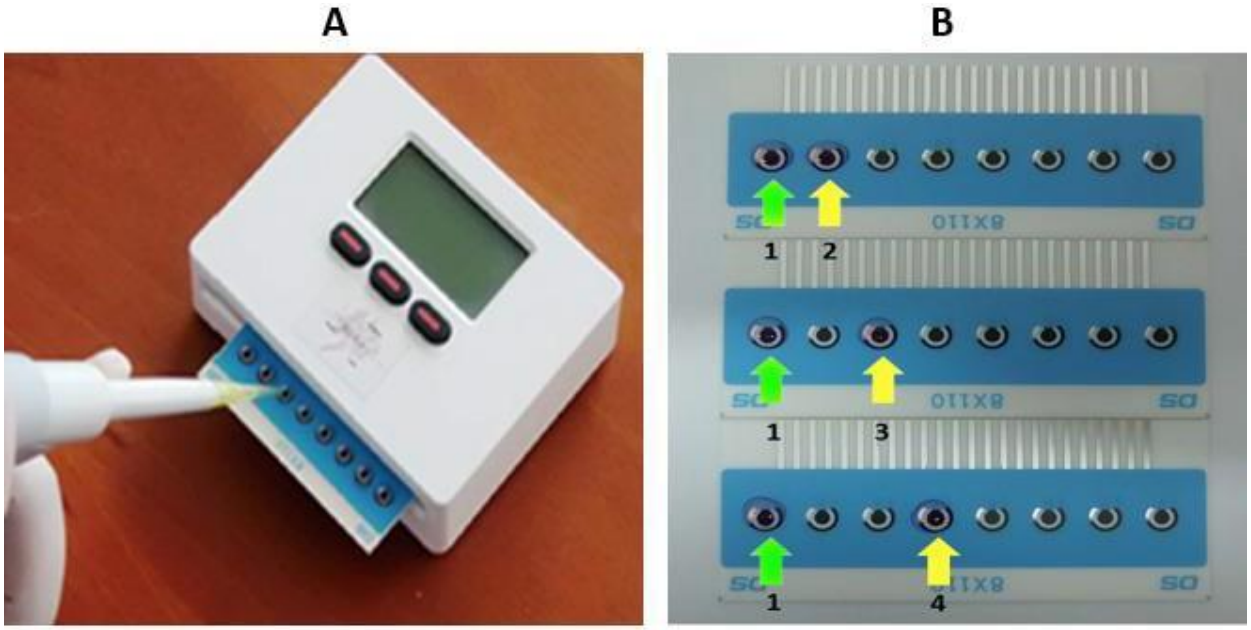


***Figure 27**. The biosensor set-up for monitoring real-time distance cell response synchronization: **(A)** The adapted multi-channel potentiometer with an eight-channel disposable electrode strip. The neurotransmitter solution (100 μM dopamine) is added to the inducer cell suspension (Position 1) by means of an automatic pipette. **(B)** Green arrows indicate the inducer (dopamine-treated SK-N-SH cells) cells and the yellow arrows indicate the detector (untreated) cells in different measurement channels (electrode pairs) in order to investigate the effect of NCDCI signaling. Inducer cells are always positioned at position 1, while detector cells are positioned either at position 2, 3 or 4.*

In the context of the present study, the following four experiments were performed:

During the first experiment, the response of the detector cells located at position 2, either at 3 or 4 (Figure 27B) was recorded, after the inducer cells located at position 1 were stimulated with dopamine.

In the second experiment, the same experimental procedure was followed but this time no dopamine was added to the inducer cells in order to investigate the possibility of NCDCI being present in the cells in the absence of a population of inducer cells.

In the third experiment, the inducer cells placed at Position 1 were separated from the detector cells located at Position 2 by different barriers in order to investigate the involvement of electromagnetic phenomena during cellular signaling. In the fourth experiment, inducer cells (Position 1) were first incubated with vinblastine and then

stimulated with dopamine and the response of inducer cells located at Position 1 and detector cells located at Position 2 was recorded. The same experiment was repeated with the detector cells (Position 2) this time incubated with vinblastine. This was done in order to investigate the possibility of the involvement of UV on NCDCI.

Imaging actin and tubulin cytoskeleton

For cytoskeleton microscopical analyses cells seeded at a density of 5 x 10^4 were cultured overnight and then were fixed for 20 min with PBS containing 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 and 5% BSA for 1 hour at room temperature. Cells were incubated with Anti-mouse α-tubulin (1:500) for 1,5 hour. Subsequently, the cells were washed three times for 20 minutes and incubated with the secondary antibody (binding to mouse α-tubulin) and 200 nM phalloidin Atto 438 for 2 hours at room temperature. Finally, the samples were washed thoroughly and then placed in 10 ng/ml DAPI fluorescent support. Photographs were taken on a fluorescence microscope (Zeiss, Germany) equipped with Image Pro Plus software.

Experimental design

The experimental design was followed by a completely randomized design, where two replicates were performed for each different cell (detector-inducer) configuration (n = 360 per replicate). Each experiment was repeated 5 times in total on different days. The data were normalized to allow comparisons of measurements made on different dates. The differences between the time-series of the potentiometric measurements recorded at different locations were statistically approximated using a t-test assuming unequal variations.

3.4 Results

Synchronization of responses between inducer and detector cells depends on distance

The new biosensor system developed was capable of directly recording changes occurring in the membrane potential of inducer 1 cells and detector cells in either the adjacent measurement channel (Position 2) or in the farthest channels, located at Positions 3 and 4. Thus with this arrangement, we were able to directly compare the response of the inducer cells to that of the detector cells. The experimental setup, as described above, enabled us to find that both the inducer cells and the detector cells were under similar experimental conditions during the measurement, with the only difference being the exposure of the inducer cells to dopamine. At this point, it would be appropriate to note that measurements were made simultaneously at all electrode positions (Positions 1-8), but no responses were recorded in non-cell positions (Positions 5-8). Responses to positions other than Position 1 were recorded only when detector cells were placed at specific positions on the electrode as described in section 2.3.

In the context of the first experiment, a similar pattern was observed in the response of inducer cells (treated with 100 µM dopamine) to Position 1 compared to the detector cells (exposed to dopamine) located in Position 2. This was substantially demonstrated by the in parallel, time-dependent patterns of membrane potential changes in both cell groups (Figure 28A).In a statistical sense, the observed response patterns between the two adjacent positions were rather correlated to each other (with an average coefficient $r^2 = 0.69$). Also, the average of the measurements for Position 2 in terms of synchronization in the cell response compared to Position 1 was slightly lower, statistically insignificant (Figure 29).

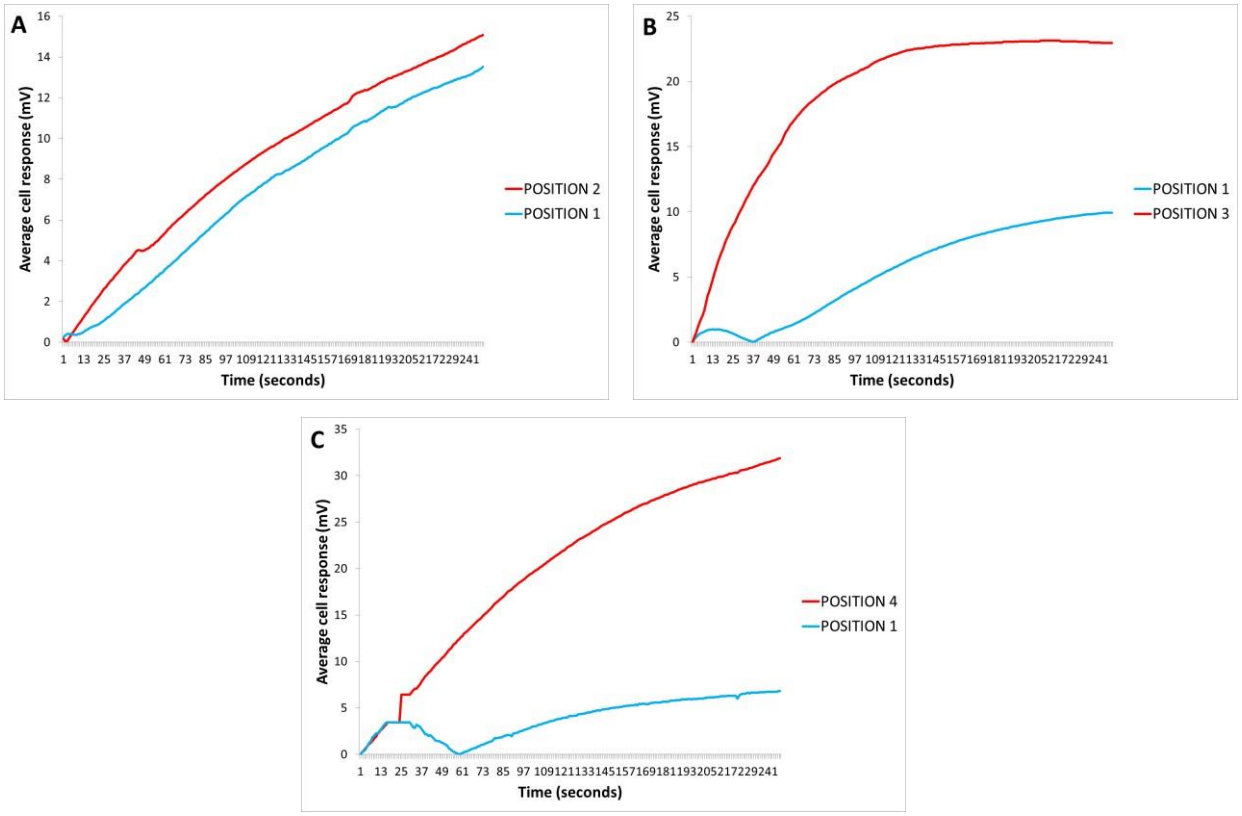


Figure 28. *The evolution of changes of cell membrane potential reveals synchronization of responses between cells treated with dopamine (inducer) and adjacent untreated ones (detector): representative, time-dependent patterns of cell membrane potential simultaneously recorded in inducer (Position 1) and detector cells in an adjacent (Position 2)* ***(A)*** *or distant positions: Position 3* ***(B)*** *Position 4* ***(C)****. Inducer cells were treated with 100 μM dopamine.*

In contrast, the responses of the detector cells recorded at the outermost Positions 3 and 4 were to a much lesser degree synchronized with the inducer cells (Position 1), as revealed by both time-dependent cell membrane potential patterns (Figures 28B, 28C), as well as from significantly lower and statistically different responses of detector cells at these positions compared to inducer cells (Figure 29). Also contrary to Position 2, recorded responses on Positions 3 and 4 were less correlated with those on Position 1 (average r^2 = 0.53 and 0.22, respectively).

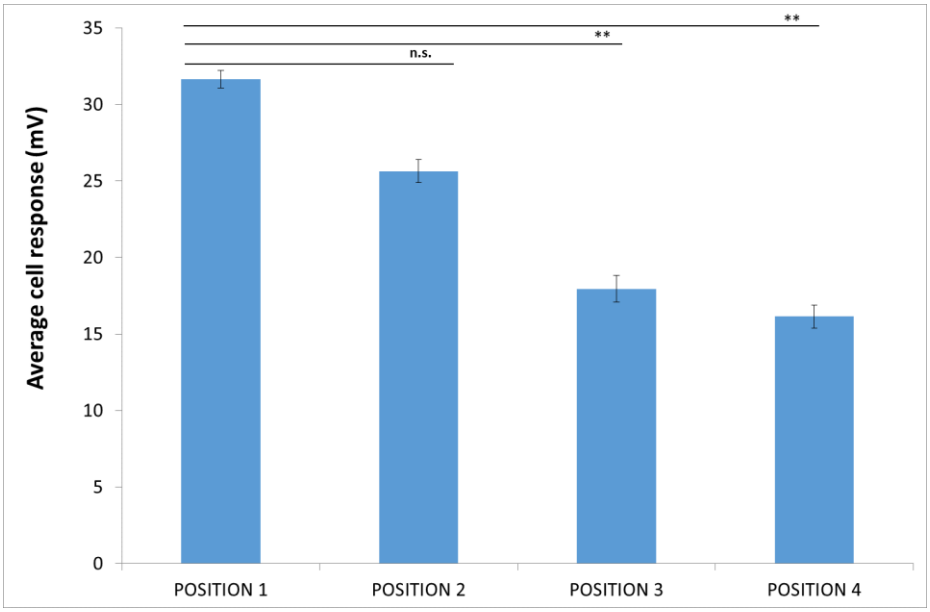


Figure 29. *The synchronization of cellular responses depends on the distance between inducer and detector cells: mean responses (change in cell membrane potential) of inducer (Position 1) and detector cells (Positions 2, 3 or 4) after addition of 100 μM dopamine at position 1. n.s = statistically non-different, ** = p<0.01 statistically different from Position 1.*

The effect of distance on response synchronization is also shown in Figure 30 (blue line), which compares the relative response differences between inducer cells (Position 1) and detector cells at different Positions (Positions 2, 3 or 4). The data presented in the figure clearly show that the response difference with respect to inducer cells increases with distance from them (from 19% at Position 2 to 43% at Position 3 and 49% at Position 4).

We have also explored the possibility of a cell-free electrode response next to the inducer cell position. There was absolutely no response in this case, which is consistent with the results from different applications using the same cell-based biosensor configuration (e.g. Apostolou et al., 2017). In simple terms, the cell-free electrode positions could not perceive by themselves the bioelectric changes in the adjacent, cell-covered positions.

Then, to test the effect of dopamine on the electrodes without the presence of cells, we added dopamine to the electrode and recorded its response. The results showed a very low response near-zero (= 0.07 $\pm$ 0.002 V) both at Position 1 where the inducer cells

were positioned and at Positions 2-4 where the detector cells were located. Therefore, we concluded that the observed results are due only to the presence of SK-N-SH cells. In other words, only the presence of cells in one position could cause changes in the response to neighbouring positions, and therefore the presence of the distant communication mechanism.

Synchronization of responses between inducer and detector cells depends on dopamine-induced cell stimulation

In the context of the second experiment, no dopamine was present in the solution of the inducer cells located at Position 1 in order to have exactly the same conditions as the detector cells. In this case, there is no recognizable pattern of differences in the responses of cells located in different positions. Differences in the response of detector cells (Positions 2-4) with respect to inducer cells (Position 1) range from 46% (Position 2) to 15% (Position 3) to 27% (Position 4) (Figure 30, orange line). In other words, no clearly identifiable NCDCI pattern is observed, in the absence of dopamine-induced stimuli.

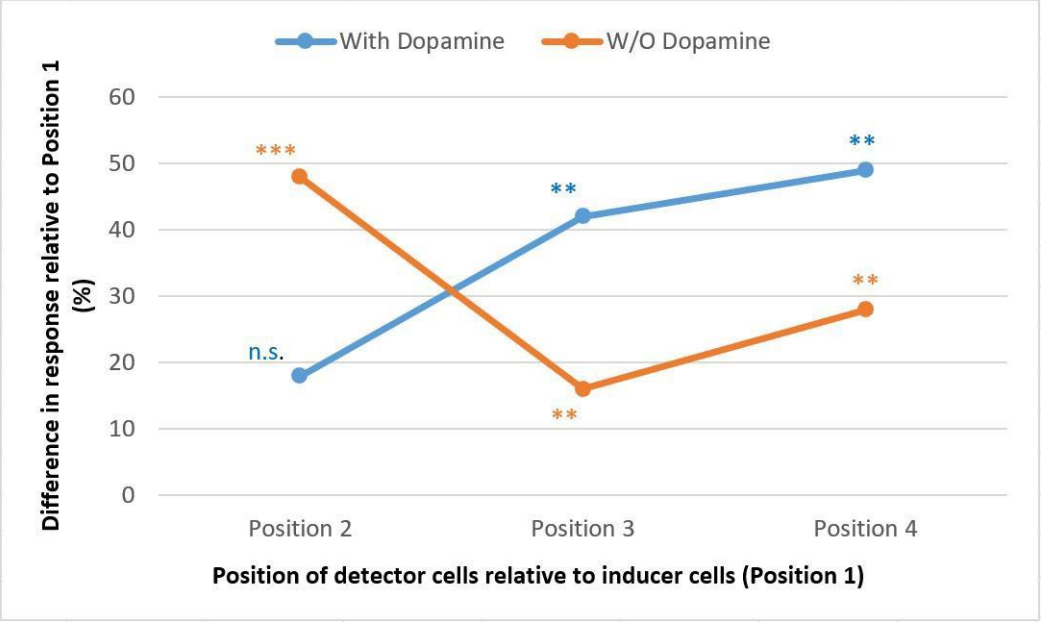


Figure 30. *Synchronization of cell responses depends on the presence of a stimulus: Differences in responses between inducer cells (Position 1) and detector cells (Positions 2, 3 or 4) expressed as a percentage of the change (in absolute values) of the detector cell responses relation to the response of inducer cells. Inducer cells were either treated with 100 μM dopamine (Experiment*

*1, blue line) or not (Experiment 2, orange line). n.s = statistically non-different, ** = p<0.01, *** = p<0.001 statistically different from Position 1.*

Synchronization of responses between inducer and detector cells depends on the barrier's material

In the third experiment, the inducer cells (Position 1) were separated from the detector cells (Position 2) by different barriers. The barriers had different spectral transparency between them because this is the only indicator of the involvement of electromagnetic phenomena during cellular signaling, and in order to avoid changes in the surface or chemical properties of the environment, the barriers did not come into contact with the cells. This has resulted in all cells being in constant contact with the same surface thus excluding any chemical or physical effects due to different surfaces. All the relevant parameters such as the chemical properties of the media and the temperature remained the same for all samples.

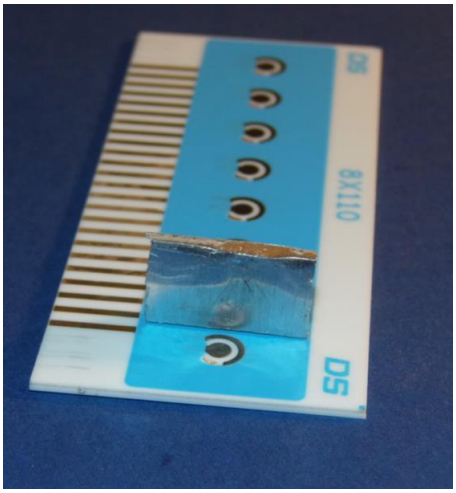
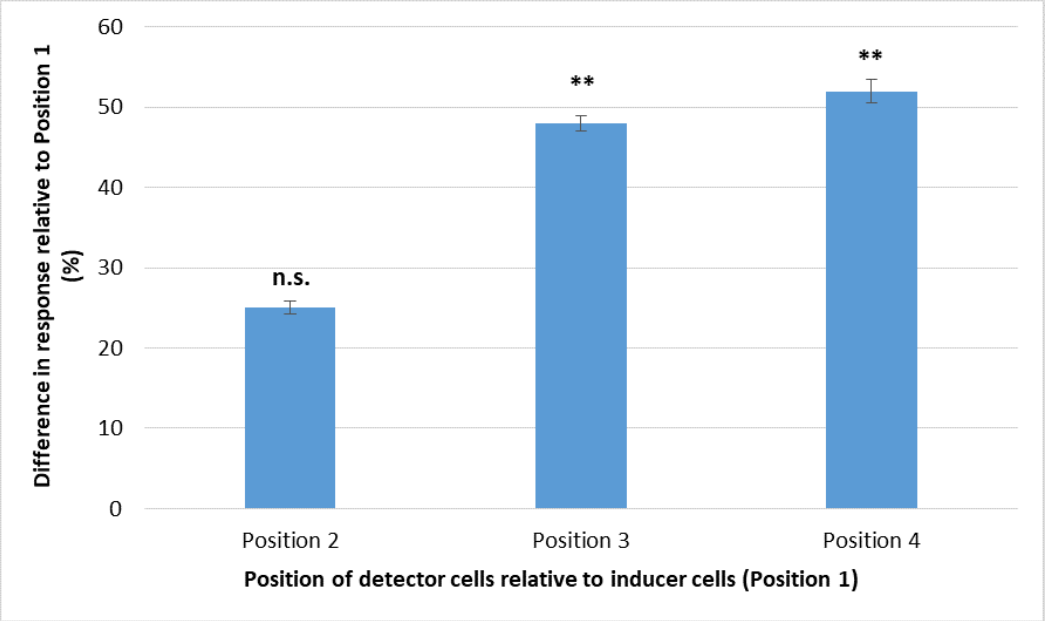


Figure 31. *Eight-channel disposable electrode strip with a metal barrier, that separates inducer cells from detector cells.*

To investigate the nature of the mechanism that involved NCDCI, inducer cells were separated from the detector cells with a plastic or a metal barrier (Figure 31). As previously, the results of the measurements were processed to express the difference in responses between inducer cells (Position 1) and detector cells (Positions 2, 3 or 4), expressed as a percentage of the change (in absolute values) of the detector cell responses related to the response of inducer cells. In the experiment where cells were separated with a plastic barrier, slightly identical patterns of response compared to control experiment (Figure 30, blue line) were revealed, but this time the response difference with

respect to inducer cells increased with the plastic barrier from 19% to 25% at Position 2, from 43% to 48% at Position 3 and from 49% to 52% at Position 4 (Figure 32A). As shown from the results, detector cells had an increase at the difference in response compared to the control experiment meaning a less synchronization on change in electrical potential between inducer and detector cells.

A



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B

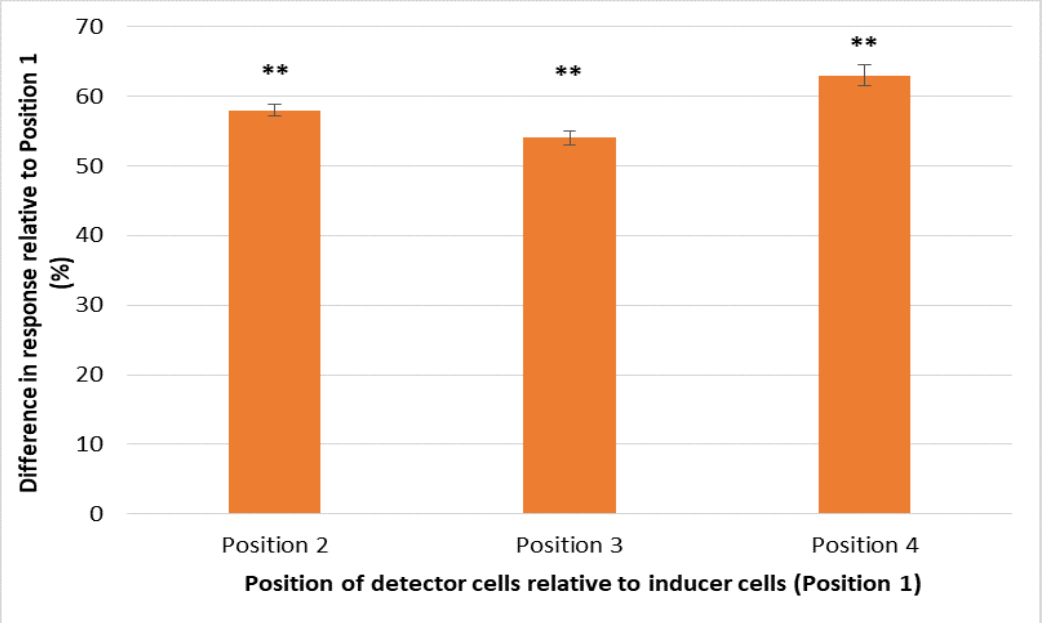

Figure 32. *Synchronization of responses between inducer and detector cells depends on the barrier's material. Differences in responses between inducer cells (Position 1) and detector cells (Positions 2, 3 or 4) expressed as a percentage of the change (in absolute values) of the detector cell responses relation to the response of inducer cells. Inducer cells were separated from detector cells by a plastic barrier* ***(A)*** *or a metal barrier* ***(B)****. n.s = statistically non-different, ** = p<0.01 statistically different from Position 1.*

Then the same experiments were repeated with a metal barrier this time (Figure 32B). In this case, we clearly see that the presence of the metal barrier affected the measurements on the adjacent electrodes, especially in the electrode at Position 2, unlike the previous results. In fact, the presence of the metal barrier gave the biggest difference in response in Position 2, compared to what was expected. Those results drive us to the hypothesis that probably a mechanism based on electromagnetic field is involved in NCDCI.

Moreover, to examine this theory, the same procedure as above was followed but this time cells were separated by a glass or quartz barrier. Glass and quartz barriers were used a) because they have different transmission spectra b) to help separate cells at the molecular rather than electromagnetic level and c) to obtain information about the frequencies of the communication phenomenon, that is, whether due to ultraviolet light or waves that are farther from the ultraviolet light.

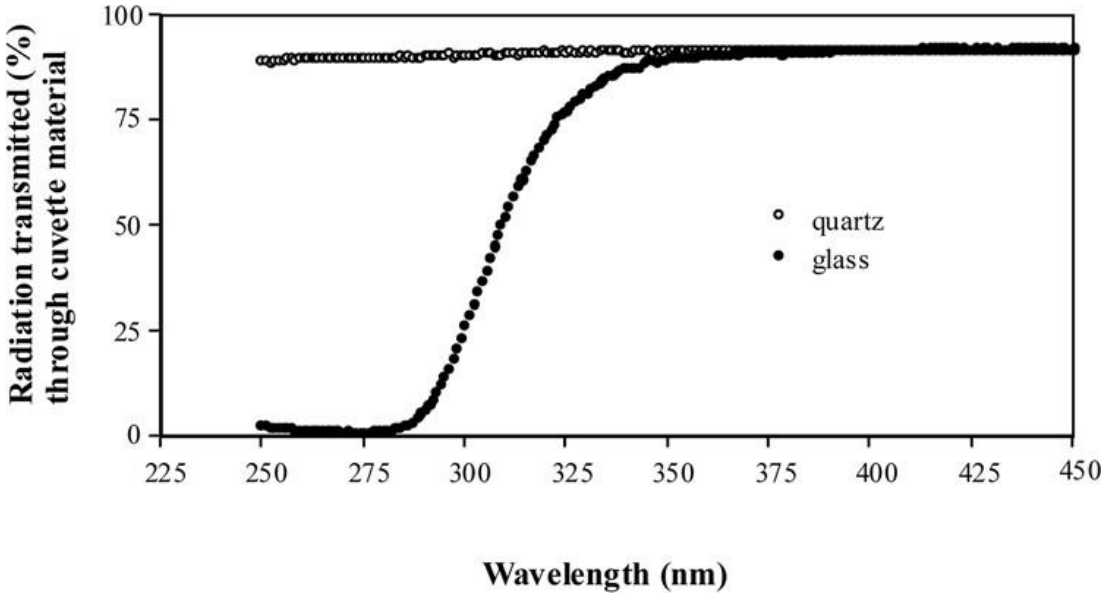

Figure 33. *Transmission of electromagnetic waves through glass and quartz. The graph shows the difference in wavelength transmission between glass and quartz. (Adapted by doi:10.1371/journal.pone.0005086.g001).*

In addition, the quartz transmits radiation at wavelengths greater than 150 nm and the glass allows high light transmission below 340 nm, so the glass serves as a UV filter (Figure 33). The results are expressed as a percentage (%) of the difference from the control (no filter). We considered that any difference of more than 20% indicates an obstruction. The results (Figure 34) showed that the smallest difference was observed when the quartz barrier was used to separate inducer cells (Position 1) from detector cells (Position 2). The difference with the control was very small (approx. 7%) compared with the glass with a difference approximate 60%. Therefore, there is evidence of UV involvement.

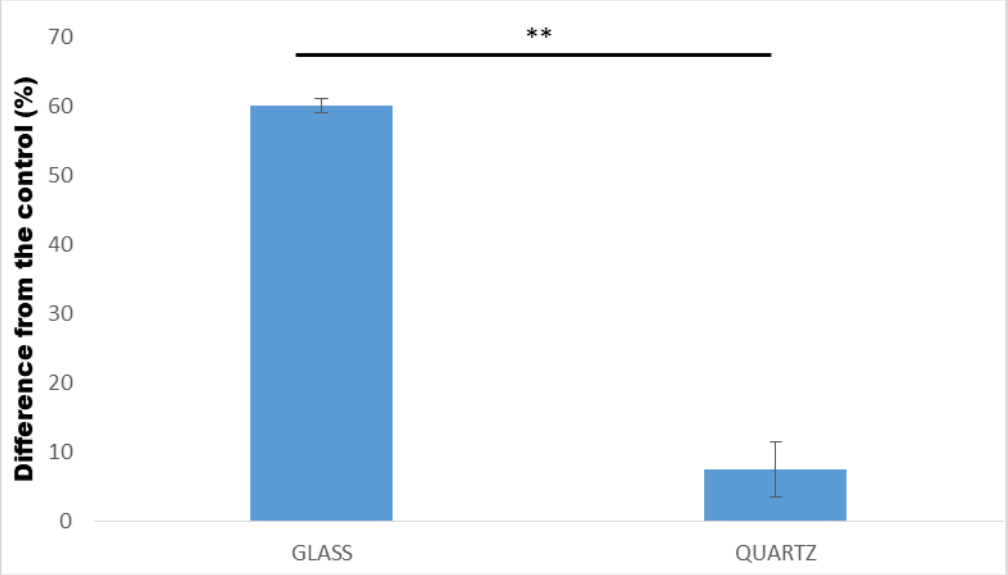

Figure 34. *Synchronization of responses between inducer and detector cells depends on the barrier's material: Results are expressed as a percentage (%) of the difference from the control (no filter).Inducer cells (Position 1) were separated from detector cells (Position 2) by a glass or a quartz. ** = p<0.01 statistically different.*

Estimation of the effect of Vinblastine – microtubule inactivation-

Tubulin was congregated in microtubule geometries, and evidence has shown that it can support energy transfer, which could be important for biological signalling and communication. Microtubules have been shown to reorganize in a dose-dependent manner after exposure to ultraviolet light, with the greatest effect observed at around 280 nm. Such a signalling mechanism may explain the observed apparent UV mediated cell-to-cell communication. Only further research, both theoretical and experimental, will tell. In the fourth experiment, inducer cells (Position 1) were first incubated with vinblastine 5μM and then stimulated with dopamine and the response of both inducer cells (Position 1) and detector cells (Position 2) was recorded. The same experiment was repeated with the detector cells (Position 2) this time incubated with vinblastine. Results are expressed as a percentage (%) of the difference between inducer cells at Position 1 and detector cells at Position 2. This was done in order to further investigate the possibility of the involvement of UV in NCDCI.

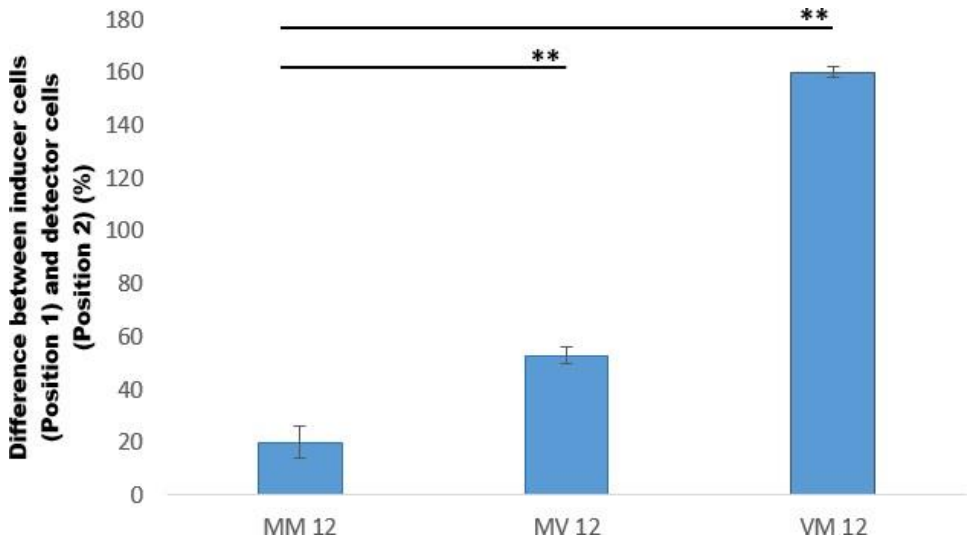

Figure 35*. Estimation of the effect of Vinblastine: Results are expressed as a percentage (%) of the difference between inducer cells at Position 1 and detector cells at Position 2. In all three experiments, 100mM dopamine was added in the inducer cell suspension. Control experiment (MM 12) was compared with the experiments where the detector cells (MV 12) and inducer cells (VM 12) were incubated first with Vinblastine respectively. ** = p<0.01 statistically different.*

This hypothesis is supported by the results of experiments with microtubule inhibition (known to be pulsed in the ultraviolet spectrum) with the addition of vinblastine. As shown in Figure 35, the addition of vinblastine to the inducer cells (VM12) increased the difference between the two electrodes relative to the control (MM12) vertically, while the addition to the second electrode (detector, MV12) also had an effect, albeit less in intensity (obviously the prevention of induction is more important than the detection).

Actin and microtubule cytoskeleton imaging after incubation with Vinblastine

In this experiment, we aimed to investigate if the concentration of 5μM Vinblastine that was used on the previous experiment was capable to inhibit microtubules, so to verify our hypothesis for UV involvement. The effect of 5 μM Vinblastine treatment on cell skeletal components was investigated. To this end, actin and tubulin filaments were analyzed in SK-N-SH cells by immunofluorescence assays (Figure 36).

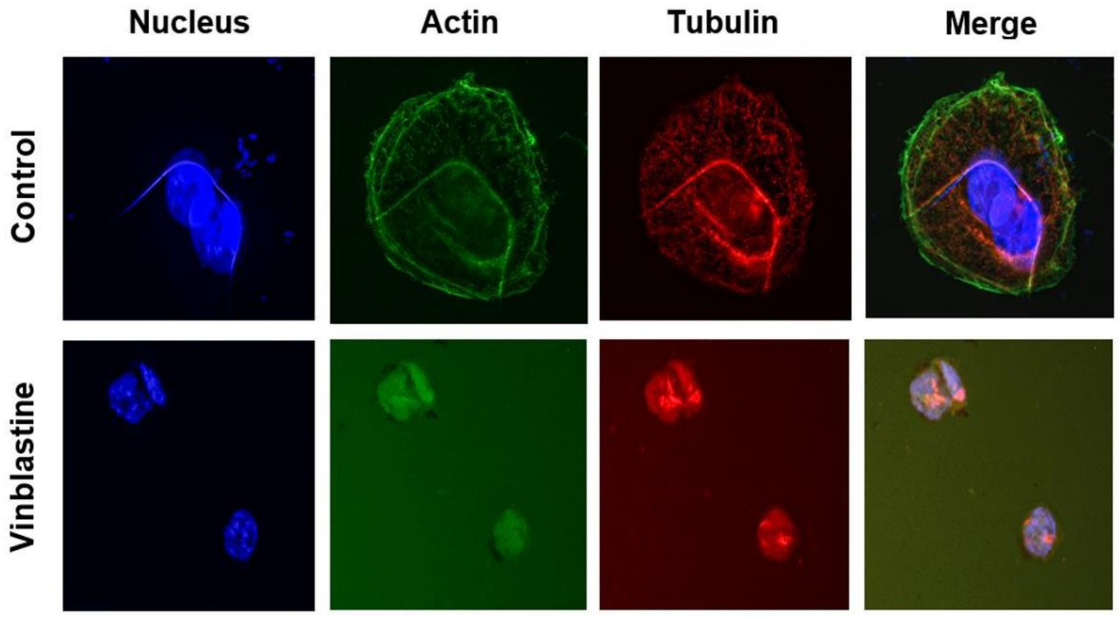

Figure 36. *Alterations of cellular actin and tubulin cytoskeleton in Vinblastine-treated cells. Tubulin was visualized with an anti-α-tubulin antibody (red), actin was visualized with Atto 438 phalloidin (green) and the cell nucleus was visualized with DAPI (blue).*

As expected, untreated (control) cells exhibited significantly fewer changes in actin and tubulin localization, with diffuse actin distribution throughout the cell compared

to the significant condensation of actin induced by 5μM of vinblastine incubation. Indeed, these results showed that the concentration which was used in the previous experiment was enough to inactivate microtubules. In conclusion, these results further strengthened our hypothesis of the involvement of UV light and possibly microtubules on NCDCI.

3.5 Discussion

In this work, we investigated for the first time the phenomenon of non-chemical, distant communication between cells of the same type using human neuronal cells in parallel with the addition of the neurotransmitter dopamine which served as the method of determining the inducer cell population. With this experimental setup, it was possible for the first time to observe the presence of NCDCI in the coordinated responses of neuronal cell populations due to their stimulation by a common neurotransmitter.

The new biosensor assembly used in this work provided us with significant advantages in the study of the existence of NCDCI among SK-N-SH neuroblastoma cells. The most important was the real-time monitoring of cell membrane changes due to the synchronization of responses between inducer and detector cells. This is a significant breakthrough compared to existing reports (Farhadi et al., 2007; Chaban et al., 2013; Hashemibeni et al., 2014; Fels, 2016) where the existence of distant communication was assessed with a series of biochemical assays, such as measuring cell proliferation which takes hours to days after hypothetical presence of NCDCI, analysis of NFκB and $[Ca^{2+}]i$ activity, the total protein content, and evaluation of cytoskeletal structure. Consequently, any changes in the response or structure of the detector cells, due to the stimulation of inducer cells, were observed much later than the hypothetical signaling event. In contrast to existing experimental approaches, our approach reduced the occurrence of such errors due to delayed observation of the physiological processes likely associated with the existence of distance communication.

In the present work, we have demonstrated the possible existence of communication between two populations of SK-N-SH cells, which were physically separated from each other, thus preventing the occurrence of any exchange of chemical signals by the solid or liquid medium. The appearance of NCDCI was more evident after stimulation of the dopamine inducer cells, although the appearance of synchrony in the

cell response did not extend beyond the neighboring detector cell group (Position 2), i.e., a few millimeters.

Additional experiments for elucidating the nature of the distant communication between SK-N-SH neuroblastoma cells were carried out. Experiments based on the evaluation of different barriers between two cell populations located in isolated neighboring positions were investigated. Observed results were then compared to control experiments. The results of these experiments demonstrated a considerable difference in the potential rate between the detached cultures when a barrier, which is non-transparent in a certain frequency region, was located between adjacent positions. This shows that the interaction is contingent on the electromagnetic emission from the cell cultures.

Indirect evidence exists that biophotons act as carriers of information, indicative of the different effects stemming from the segregation of cell populations through the use of quartz or glass. It is proposed that cell cultures use ultraviolet light for cellular information transfer, which at least affects energy uptake (Fels, 2009). There is evidence to suggest that tubulin agglomerated in microtubule geometries can support energy transfer, which may be important for biological signaling and communication. Further experiments with the use of Vinblastine, a microtubule- inactivating compound, showed a significant difference between two cell cultures placed in neighboring positions (Position 1 and Position 2) compared to control experiments.

The purpose of this work was to use the new biosensor set-up we built, to investigate the possibility of non-chemical, non-electrical communication between nerve cells. The obtained results reinforce the hypothesis of the existence of distant communication between naturally-separated human neuroblastoma SK-N-SH cells. Given the fact that this communication was observed as coordinated patterns of changes in cellular membrane potential, it can be hypothesized that the observed NCDCI may be of partial electromagnetic origin. Electromagnetic waves (biophotons) are probably the most attractive candidate.

The evidence for the transmission of electromagnetic information is strong and it is difficult to think of alternative mechanisms that could have the same effects. It is worth noting that, using the new biosensor system, we have recently demonstrated that the addition of 100 μM dopamine to mouse N2a neuroblastoma cells caused cell membrane

hyperpolarization, which in turn is responsible for generating strong localized electric fields. Previous work (Fröhlich and McCormick, 2010) has experimentally supported the hypothesis of the contribution of endogenous electric fields to the coordinated responses of neural networks in vivo, while an electromagnetic nature of consciousness, as in the case of electromagnetic field theory, has also been proposed (CEMI) (McFadden, 2013; Jones, 2017). On the contrary, the experimental setup used in this work does not ensure that volatile compounds are not transferred between the cell populations. The likelihood of a distant gaseous cell-to-cell chemical interaction has previously been reported in accordance with the reported effect of CO_2 on yeast cultures (Fels, 2016; Volodyaev et al., 2013). Nevertheless, given the results of the present work, it is relatively difficult to propose a volatile compound responsible for the observed NCDCI phenomenon, given the very small range of observed effect. Advances in biophoton research are of paramount importance to the scientific community, because, as a non-invasive method, it can provide us with immense and important information regarding the non-molecular regulation of life processes. By making significant efforts in this area of research, we will someday be able to develop a non-invasive application technology that could have a significant impact on healthcare and medicine.

Chapter 4

General discussion and conclusions

Neurotransmitters are a group of chemicals released by nerve endings that allow signal transmission between neural cells. They can also be named as a chemical messenger or chemical transmitter because of their ability to transmit nerve impulses from one cell to the next throughout the nervous system. They play an important role in shaping everyday life and functions.

This doctoral thesis was divided into two sections. The first section deals with the study of the in vitro interaction of dopamine neurotransmitters with dopamine receptor-bearing nerve cells for the purpose of evaluating different drug targets, and the second section deals with the examination of the existence of non-chemical, distant cellular interactions between neuronal cells.

One of the most important aspects of brain function is dopamine signaling (DA). Many side effects of major diseases and pathologies, as well as the basis of their treatment, are based on the pharmacological modification of the response of the various dopamine receptors and its subtypes (Abraham et al., 2014). Without diminishing the importance of receptor affinity studies, another area that is equally important in neuropharmacology research is the functional assays for testing the activity of different drugs that have agonistic or antagonistic activity in relation to DA.

Previous studies (Grace & Bunney, 1984; Missale et al., 1998) have shown that different DA receptors have different DA affinity. For example, D1-like receptors have the ability to induce depolarization of the cell membrane because they stimulate cAMP accumulation while D2-like receptors induce hyperpolarization of the cell membrane as they inhibit cAMP accumulation. Consequently, the observed results reveal a preference for DA in specific receptors depending on the concentration of DA: (a) at low concentrations D1 receptors (nanomolar range) are activated and (b) at high concentrations D2 receptors are activated (micromolar range) (Furman et al., 2009).

In the first section, we reported the development of a completely new concept of analysis, in order to study, in vitro, the interaction of dopamine with dopamine receptor-bearing nerve cells. As shown in **Chapter 2**, two different patterns, depending on the DA concentration range, were demonstrated by the biosensor response, after analyzing the potentiometric measurements: (a) an increase in the cell membrane potential proportional to the increase in DA concentration between the concentrations 0-100 nM, indicating membrane depolarization and (b) a decrease in membrane potential, inversely

proportional to the increase in DA concentration, between 1-1000 μM concentrations, indicating membrane hyperpolarization.

In addition, the novel biosensor approach was used to study cell membrane potential changes induced by the presence of eticlopride, which has been described as a selective D2-like receptor inhibitor. As expected, because of the ability of eticlopride to block D2-like receptors, depolarization of the cell membrane was observed by shifting values to positive values

The novel approach was additionally used to evaluate the dopaminergic activity of chaste tree extracts (*Vitex agnus-castus*) as well as the activity of two well-known antipsychotics, haloperidol, and olanzapine which act as a D2-receptor antagonist with lower affinity than eticlopride. Extracts of this plant have been shown to be helpful in treating various pathological conditions due to abnormal dopamine concentrations such as cyclical mastalgia (Carmichael, 2008), premenstrual mastodynia, cycle irregularities (Wuttke et al., 2003), mood alteration, headache and bloating (Liu et al., 2004).

The findings of this section highlighted the importance of the development of a fast, highly sensitive high-performance screening system for DA agonists and/or antagonists, to evaluate their effect on whole cellular responses in vitro. Further, as demonstrated in **Chapter 2**, the assay can be easily combined with several advanced biosensor (mainly electrochemical) techniques to rapidly determine DA concentration after administration of agonist or antagonist molecules (Jackowska & Krysinski, 2013), thereby significantly increasing the number of information that can be retrieved from a single in vitro experiment. As has also been shown, in the case of immature *V. agnus- castus* fruit extracts, this bioelectric approach could very well be a useful tool for rapidly assessing the dopaminergic potency of natural bioactive drugs to be used in clinical trials or ethnobotanical studies.

The second section focused on the investigation of the mechanism of cell-to-cell communication and the coordinated cellular responses they present.

The first experiments related to the principle of non-chemical non-electrical communication between biological systems were carried out by Gurvitch in 1920. Gurvitch had shown that onion root cells that were chemically isolated from each other, when located close to actively dividing cells, showed increases in the number of mitoses. During the last two decades, several experiments have been carried out that have

demonstrated the existence of non-chemical communication between cells naturally separated from each other (Wainwright, 1998; Nikolaev, 2000; Trushin, 2003, 2004; Fels, 2009; Cifra et al., 2011). Various terms have been proposed to describe these phenomena with the predominant being non-chemical, non-electrical (NCNE) signaling, and non-chemical, distant cellular interactions (NCDCI) (Wainhright, 1998; 2014).

In **Chapter 3**, we investigated the existence of NCDCI among human SK-N-SH neuroblastoma cells using a new biosensor that allowed real-time monitoring of the bioelectric properties of physically separated cells. In one group we had the "inducer" cells that were stimulated with dopamine, and in the other group, we had the "detector" cells. We show that changes in membrane potential due to the addition of dopamine to inducer cells can be detected almost immediately by neighboring cells (detector cells) which in this case are not exposed to dopamine and are naturally isolated from the inducer cells, to exclude chemical exchange phenomena.

Scientists are now investigating the existence of a new form of light communication, after several studies revealing that living cells not only emit light but they also absorb light and process the information associated with it.

Several researchers have hypothesized that communication must take place at the speed of light in order to enable the living processes to be organized. Biophotons could explain the existence of a new type of communication, complementary to the already known chemical and electrical communications (Popp & Zhang, 2000). According to Sun (2010), photosensitive biomolecules of neurons and cells are capable of absorbing biophotons and in turn transfer the energy resulting from the absorption of biophotons to nearby biomolecules, thereby causing changes on the signal processes between cells.

Recently another work has examined the possible existence of distant communication with the help of biophotons after demonstrating synchronization in the development of fish eggs (Mayburov, 2011). At the same time, some scientists proposed that the brain could be the ideal place for photon communication. In fact, microtubules could function perfectly as optical fibers for the transmission of biophotons into the brain nerve cells (Grass et al., 2004). Most researchers now argue that the role of biophotons in the brain deserves particular attention (Rahnama et al., 2011). Verifying the electromagnetic interaction between nerve cells would significantly increase the evidence

for a neuro-electromagnetic field capable of interacting with the brain to form a unified field of consciousness (Jones, 2017).

In order to investigate the nature of the mechanisms that cause the observed distant cell interactions, additional experiments were carried out. Experiments based on the evaluation of different barriers between two cell populations located in isolated neighboring positions were investigated. Observed results were then compared to control experiments. The results of these experiments demonstrated a considerable difference in the potential rate between the detached cultures when a barrier, which is non-transparent in a certain frequency region, was located between adjacent positions. This shows that the interaction is possible contingent on the biophotons emission from the cell cultures.

Advances in biophoton research are of paramount importance to the scientific community because they cannot only give us important insights into understanding why human beings could hold such a high stake in our intelligence, but also provide new ideas for developing artificial intelligence products and functional brain models. In parallel, as a non-invasive method, it can provide us with immense and important information regarding the non-molecular regulation of life processes. By making significant efforts in this area of research, we will someday be able to develop non-invasive application technology that has a significant impact on the nature of healthcare and medicine.

Chapter 5

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