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Mini Review

# The biological networks in studying cell signal transduction complexity: The examples of sperm capacitation and of endocannabinoid system

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ABSTRACT

Cellular signal transduction is a complex phenomenon, which plays a central role in cell surviving and adaptation. The great amount of molecular data to date present in literature, together with the adoption of high throughput technologies, on the one hand, made available to scientists an enormous quantity of information, on the other hand, failed to provide a parallel increase in the understanding of biological events. In this context, a new discipline arose, the systems biology, aimed to manage the information with a computational modeling-based approach. In particular, the use of biological networks has allowed the making of huge progress in this field.
Here we discuss two possible application of the use of biological networks to explore cell signaling: the study of the architecture of signaling systems that cooperate in determining the acquisition of a complex cellular function (as it is the case of the process of activation of spermatozoa) and the organization of a single specific signaling systems expressed by different cells in different tissues (i.e. the endocannabinoid system). In both the cases we have found that the networks follow a scale free and small world topology, likely due to the evolutionary advantage of robustness against random damages, fastness and specific of information processing, and easy navigability.



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## 1. Introduction

The ability to respond to external and internal stimuli is a key propriety of living cells. Continuously, every cell in multicellular organisms, receives a myriad of messages from itself, from other cells, and from the surrounding environment, elaborates them, and produces an output response. These messages could be either of chemical (signaling molecules such as proteins, lipids, ions, $O_2$, pH) or physical nature (action potential, mechanical or thermal stress, photons, etc…). This, on the one hand, assures an amazing ability to detect and to adapt the cellular biochemical machinery to a very broad spectrum of situations, in order to maintain the cellular homeostasis, on the other hand, it causes the emergence of a complex pattern of behavior. Indeed, it is possible to define complex phenomenon which emerges from a collection of interacting objects [1], exactly as it happens when molecules and cells interact with each other generating different signal transduction pathways. The study of complexity, in very different fields of sciences, led the researchers to fix some common aspects. [1,2].

### 1.1. Biological complex systems are constituted by interacting objects

In cell biology, for instance, the cells exchange messages with themselves (autocrine communication), with the surrounding cells (paracrine communication), or with cells located in other tissues (endocrine communication). Interestingly, these networks of molecular messages could involve also the dialogue with other individuals of the same or different species (social communication) as it is the case of pheromones or chemoattractants.

### 1.2. The behavior of the objects composing the systems is affected by memory (the so called Hysteresis)

Cellular systems are dynamical entities, evolving with the time, and prior states have an influence on present states. This characteristic is on the basis of the ability of signal transduction cascades to adapt their strategies according to their history. This is a very important propriety of cellular communications and, in general, of living systems. It allows cells to be able to learn from their experience and to mold their response in function of contingent situations.

### 1.3. Complex systems are typically open

If it is evident that cells exchange matter and energy with the environment in which they live, it is also evident that a continuous flux of molecules from and to living beings is on the basis of their survival.

### 1.4. They give rise to emergent phenomena, whose entity could be extreme

Often, the system response to a stimulus is not predictable and it will not be proportional to the intensity of applied solicitation. This is the reason why it is impossible to accurately predict the behavior of a cellular system to an input, and why small perturbation could lead to dramatic consequences while large ones could exert only negligible effects. Sometime, this feature of complex systems is ascribed to the so called "butterfly effect", referring to the idea that a butterfly wings might create tiny changes in the atmosphere that may, ultimately, accelerate or even prevent the occurrence of a tornado in another location [3].

Biologists are greatly interested to the consequences of the complex behavior of cellular systems. In particular, the most relevant problems are, firstly, the intrinsic impossibility to perform reliable prediction about the biological systems evolution after a sufficiently long time interval. Indeed, every finished model representing the initial status of a system needs to remove some information about the initial conditions. This determines an error that tends to increase as the simulation proceeds over time. At the limit, the residual error in the simulation could be of the same order of magnitude than the result itself: in this case the predictions from the simulation are no longer reliable. In addition, changing the scale by which the system is observed, new and unexpected proprieties will emerge. It is, for instance, the case of neuronal networks: each neuron per se obeys to a few simple rules (ultimately the activity of each neuron in terms of action potential could be represented by two states: on or off), while a large number of them, reciprocally interacting, is able to display a complex and sophisticated behavior, such as memory, learning, imagination, or creativity.

The need to face with biological complexity has prompted scientists to use two different approaches: the Reductionism or the Holism. The Reductionism is a way to understand the nature of complex things by reducing them to the ensemble of their parts, or to simpler or more fundamental things. Thus a "reductionist believes that a complex system is nothing but the sum of its parts. An account of it can be reduced to accounts of individual constituents" [4]. On the contrary holists think that in a complex system the whole system is more than the sum of its single components. This concept is elegantly pointed out by the etymology of the word "complexity": it derives from Latin *cum* = together and *plecto* (that in turn derives from the ancient Greek πλέκω) = plait, weave, braid, twist, and turn; thus giving the idea of interconnected and not separable things. Noteworthy the difference of meaning between the words "complex" and "complicated" is evident. "Complicated" derives from the Latin *cum* and *plicare* (to fold); the solution of a complicated problem is the explication (from Latin *explicare* = to unfold), on the contrary it is impossible to "unfold" the complexity.

Recently, we assisted to an enormous increase in the amount of information about cells molecular biology, due to the even more extensive application of high throughput technologies, such as 2D electrophoresis, DNA microarrays, and protein chips, and new branches of science (genomic, epigenomic, lipidomic, proteomic, metabolomic) have originated, all converging in the study of systems biology. In this context the holistic approach is, in the opinion of several researches, essential. As Marc H. V. Van Regenmortel affirmed, the situation of reductionists in face of biological complexity "is similar to that of an art student asking about the significance of Michelangelo's David and being told that it is just a piece of marble hewn into a statue in 1504. This is certainly true, but it evades pertinent questions about the anatomy of the statue, its creation at the beginning of the Florentine Renaissance, its significance in European art history, or even the scars on its left arm that were plastered after it was broken in three places during the anti-Medici revolt of 1527. In an analogous way, the biology, development, physiology, behavior or fate of a human being cannot be adequately explained along reductionist lines that consider only chemical composition" [5].

## 2. The networks to study complex phenomena

The need to taking in account the complexity of cell biology in analyzing signal transduction pathways, together with the availability of innovative computational tools, has led several groups to adopt a computational modeling approach. Actually, the idea to use a model, and in particular a set of nodes connected by links (a graph), is no new, indeed it dates back to 1736, when Leonhard Euler solved the Seven Bridges of Königsberg problem. The city of Königsberg in Prussia (now Kaliningrad, Russia) was set on both sides of the Pregel River, and included two islands connected to each other and to the mainland by seven bridges. The problem was to find a walk through the city that would cross each bridge once and only once. The islands could not be reached by any route other than the bridges, and every bridge must have been crossed completely every time. Euler, for the first time, represented the mainland and the islands as nodes and the bridges as edges connecting them, thus originating a network. Using this modeling strategy, he provided the evidence that the problem had no solution [https://www.math.dartmouth.edu/~euler/pages/E053.html]. Now, computational models based on networks theory are widely used to study

several different phenomena, from the WWW architecture [6–8] to the physical connection of computers through the world [6], from the actors collaboration chains to the company market [9], pointing out as some similar features seem to be shared by the most of them, thus allowing the classification of networks in different classes.

### 2.1. Random networks

The simplest is that of random networks: a random network is obtained by connecting a set of $n$ nodes with randomly added edges. The most commonly studied models of random networks have been proposed by Edgar Gilbert in which every possible edge occurs independently with probability $p$ [10], and that proposed by Paul Erdős and Alfréd Rényi in which it exists an equal probability of all graphs with exactly $M$ edges (Erdős–Rényi or ER model) [11]. Two different variants of ER model are known:

- the $G(n, M)$ model, in which a graph is chosen at random from the collection of all graphs with $n$ nodes and $M$ edges. For example, in the $G(3, 2)$ model, each of the three possible graphs on three vertices and two edges are included with probability 1/3;
- the $G(n, p)$ model, in which a graph is realized by randomly connecting the nodes: each edge is added to the graph with a probability $p$, independent from other edges. Thus, all graphs with $n$ nodes and $M$ edges have equal probability of $p^M(1-p)^{\binom{n}{2}-M}$.

In random networks, the node degree (i.e. the number of links per node) follows a Poisson distribution, thus the most of nodes have approximately the same number of links, close to the average degree, that defines the network scale. The tail (high $k$ region) of the degree distribution $P(k)$ decreases exponentially, thus the nodes that significantly deviate from the average are extremely rare. The clustering coefficient is independent from the node degree, and the mean path length is proportional to the logarithm of the network size, $l \sim \log N$ [11]. From a biological point of view, random networks have two important features:

- the network behavior strictly depends on the network scale;
- random networks are democratic networks, i.e. each node concurs as all others nodes in determining network proprieties.

For instance, the water at liquid state, is constituted by several cluster of molecules aggregated to form domains, due to the presence of hydrogen bonds. Each water molecule binds on average 4 other molecules, thus it is possible to represent water as a network of scale 4.

### 2.2. Scale-free networks

Random networks are unable to describe an evident peculiarity of several biological and not biological systems: the heterogeneity of node degree. Indeed, the WWW connectivity as well as the Internet architecture, the stock market, the protein interaction networks, share a very important architectural feature: the presence of a low number of highly connected nodes (the "hubs") and a higher number of scarcely connected nodes [12]. In 1999 Albert Lazlo Barabási and Reka Albert proposed the so called Barabási–Albert (BA) model, based on growth and preferential attachment [13]. These networks are able to grow over the time, and the growth is realized by the attachment of new nodes preferentially to the hubs: the more one node is connected, the higher is the probability that it attracts a new node attaching to the network. Thus, the probability $p_i$ that the new node is connected to node $i$ is:

$$pi = \frac{ki}{\sum_j kj}$$

where $k_i$ is the degree of node $i$ and the sum is made over all preexisting nodes $j$ [13].

BA networks are characterized by a power-law distribution of node degree, i.e. the probability that a node has $k$ links follows $P(k) \sim k^{-\gamma}$, where $\gamma$ is the degree exponent, which usually ranges between 2 and 3. Differently from what happens in random networks, in these networks the network behavior is strongly determined by the small number of hubs (a sort of "super-nodes") and that a "typical" node does not exist (they have a scale-free topology). Another important characteristic of BA networks is that the clustering coefficient, $C(k)$, is independent of $k$ and the average path length follows $l \sim \log \log N$, which is significantly shorter than log N that characterizes random networks [2].

### 2.3. Hierarchical networks

In hierarchical networks the scale-free network topology coexists with high modularity and high local clustering. In these networks the clustering coefficient is a function of the node degree:

$$C(k) \sim k^{-\beta}.$$

Several networks representing important biological entities have been found to have a hierarchical topology, such as metabolic networks or gene networks [14–16].

## 3. The use of biological networks to explore cells signaling

Our group, as well as many other all around the world, has applied a biological networks-based approach to explore cell signaling in different contexts.

In the present paper, we reported our finding to highline as it is possible to use the same computational strategy to achieve different results, as a function of the described event. Indeed it has been possible to study the process of capacitation, and more in general, the post-ejaculatory activation of spermatozoa, identifying the hubs of the system and comparing organisms with different reproductive ecology. In the case of endocannabinoid system (ECS), we adopted a different approach: we studied the backbone of a signaling system expressed by different cells in different tissues, where it plays different roles, from the control of immune response to the neurotransmission, from the regulation of food intake to the modulation of reproduction. In other words, the same tool, the biological networks, could be used to explore the same function in different contexts or the same system that exerts different function in different tissues.

In both cases, since specific databases already available online did not exist, the data concerning the molecules involved and their interactions have been manually retrieved in peer reviewed articles from PubMed (http://www.ncbi.nlm.nih.gov/pubmed/) published in last 10 years. On these bases, different databases have been realized using Microsoft Excel 2003, specifying for each molecular interaction described the following fields: a) source molecule: it represents the molecule that is the source of interaction; b) interaction: it represents the nature of the connection (i.e., activation, inhibition, binding, control, degradation); c) target molecule: it represents the molecule that is the target of the interaction; d) species: it represents the species where the interaction was described; e) reference: it represents the bibliographic source of information; and f) notes: it represents all notations such as the presence of synonyms or the intracellular location (when appropriate), or the explanation of complex cellular events. Next, independent files were built up in order to define the attributes of each molecule. In particular, we considered: a) cellular localization, that is the area where the molecule is located or where it exerts its biological activity; b) pathway, that is the chain of events which the molecule belongs to.

The corresponding networks have been built up and analyzed with Cytoscape 2.8.3 and the specific plug-in Network Analyzer as directed networks (http://med.bioinf.mpi-inf.mpg.de/netanalyzer/index.php).

### 3.1. Exploring the activation of spermatozoa

In the first case, we have represented as a network the molecular events involved in the process leading mammalian spermatozoa to acquire their fertilizing ability. Indeed, immediately after ejaculation, they are unable to fertilize homologous oocyte, thus they must complete a series of biochemical modification within female genital tract, the capacitation to reach the fertilizing ability.

As first, the sperm membranes markedly change their architecture. In sperm head plasma membrane (PM) it is possible to recognize different domains, separated by diffusion barriers, characterized by different chemical–physical and functional proprieties: the apical ridge area, the pre-equatorial area, the equatorial area and the post-equatorial area. The apical ridge is involved in sperm-ZP binding and contains specific zona binding proteins [17], in the pre-equatorial surface the fusion between PM and outer acrosome membrane (OAM) occurs during AR, and the equatorial surface area is involved in the fusion between spermatozoa and oocyte at the moment of fertilization [18,19]. Each domain, in turn, contains specialized areas, known as microdomains or detergent resistant membranes (DRMs). They are small lipid ordered portions of membrane composed of large amounts of cholesterol, sphingomyelin, gangliosides, phospholipids with saturated long-chain acyl chains, and proteins such as GPI anchored proteins, caveolin and flotillin. During capacitation, their organization changes, allowing the association and activation of proteins involved in signal transduction, [20] and in membrane fusion [21,22].

The different compositions of inner and outer leaflets of sperm membranes also play an important role in the acquisition of spermatozoa fertilizing ability [23–25]. More in detail, the aminophospholipids phosphatidylserine (PS) and phosphatidylethanolamine (PE) are more concentrated in the inner leaflet of PM, while the choline phospholipids sphingomyelin (SM) and phosphatidylcholine (PC) are more abundant in the outer leaflet. This asymmetry is established and maintained by the action of several translocating enzymes [26]: the aminophospholipid translocase (also known as flippase), is responsible for transferring of PS and PE from the outer to the inner lipid leaflet, the 'floppase' transfers phospholipids from inner to outer leaflet and the scramblase acts as a bidirectional carrier with little specificity, simply moving all four phospholipids species in both directions (inward and outward) across the membrane lipid bilayer. The modulation of membrane asymmetry is a key event in controlling the ability of PM and OAM to fuse each other (fusogenicity). The lipid remodeling of sperm membranes during capacitation is controlled by activating (bicarbonate, $Ca^{2+}$, progesterone [27,28]) and inhibiting stimuli (endocannabinoids [29–31]) with the aim to coordinate sperm activation with the presence of the oocyte.

The cytoskeleton is also involved in capacitation and in particular it participates in modulation of membrane remodeling. Indeed, in spermatozoa the cytosol is virtually absent, thus the membranes are directly in contact with the underlying cytoskeleton structures. At the beginning of capacitation sperm membrane is stable, PM and OAM fusogenicity is low and in acrosomal region of sperm head the amount of polymeric actin (F-actin) is reduced. During capacitation the increase in membranes fusogenicity is paralleled by the increase in actin polymerization: the F-actin forms a network interposed between PM and OAM, thus avoiding their premature contact and fusion [32,33]. At the end of capacitation, when the proteins present on oocyte zona pellucida activate the specific receptors on sperm surface, a fast calcium peak causes the rapid depolymerisation of actin cytoskeleton, thus allowing fusion of PM and OAM and, ultimately, AR [34,35].

In addition, during capacitation, the spermatozoa change their motility pattern. Within the epididymis, spermatozoa are completely immotile or weakly motile, while immediately after ejaculation, they begin to swim with a species-specific pattern. Once exposed to capacitating condition, male gametes start to express a new pattern of motion, the hyperactivated motility. It is believed that the acquisition of this motility pattern allows the spermatozoa to penetrate the oviductal mucus, the cumuls-oocyte complex extracellular matrix and, finally, the ZP [36,37]. The hyperactivated motility is stimulated by the rise in intracellular concentration of $Ca^{2+}$ and cAMP. The $[Ca^{2+}]i$ is modulated by the activation of phospholipase C (PLC) through a heterotrimeric G protein (Gq/11)-coupled receptor (R1). cAMP is produced by the membrane-associated adenylyl cyclase (AC) through high cytoplasmic $Ca^{2+}$, G proteins and membrane potential, and by the soluble form of adenylyl cyclase (sAC) directly activated by $HCO_3^-$ ions. The increase in cAMP concentration activates cyclic nucleotide-gated channels (CNG) thus promoting the $Ca^{2+}$ influx and the protein kinase A (PKA), which phosphorylates axonemal or fibrous sheath proteins and results in flagellar beating. High cytoplasmic $[Ca^{2+}]$ and $Ca^{2+}$–calmodulin complex are responsible for asymmetrical bending of flagella that is characteristic of hyperactivation [37].

All these events are coordinated and controlled by a series of molecular signals, which, ultimately, act as a complex system. Just this complexity could be the cause of our inability to emit a reliable diagnosis in the case of male infertility, in about 50% of cases. This is the reason why we decided to adopt a biological networks-based computational modeling approach to explore the signaling system involved in capacitation. We used the molecular data about molecules known to participate in the acquisition of fertilizing ability of human spermatozoa reported in recent scientific literature to realize the capacitation networks [38]. Then, we carried out the statistical analysis of network topology. As a result, it was evident that the network representing human sperm capacitation has a scale free topology, in accordance with BA model (Fig. 1 and Tables 1 and 2).

The node size is proportional to the connection number and the node color gradient is dependent from the closeness centrality. This parameter is computed as: $C_c(n) = 1 / avg(L(n,m))$, were L (n,m) is the length of the shortest path between two nodes n and m. The closeness centrality of each node ranges from 0 (green) to 1 (red) and it is a measure of how fast the information spreads from a given node to the other nodes. The spatial network arrangement is obtained by using the Cytoscape Force-Directed Layout.

The number of nodes represents the total number of molecules involved; the number of edges represents the total number of interaction found, the clustering coefficient is calculated as $CI = 2nI / k(k - 1)$, where nI is the number of links connecting the kI neighbors of node I to each other, the network diameter is the largest distance between two nodes, the Averaged n° neighbors represent the mean number of connection of each node, the Char. path length gives the expected distance between two connected nodes.

Indeed, both the in and out node degrees follow a power law (the exponent b is respectively $-1.542$ and $-1.993$, see Table 2) and the networks have a very low clustering coefficient (0.028, see Table 1).

In our opinion, it is an intriguing finding because it could explain important biological characteristics of the capacitation process. As first, this topology confers to the capacitation of a high robustness against random damages, thus assuring the higher stability possible to a so important event. Indeed, in our network only a small number of nodes are able to establish a great number of connections (the hubs). In the most of the cases a random failure will affect the most frequent node typology, i.e. those scarcely linked, thus having negligible effects on the whole network topology. In other words, the probability that a hub will be randomly affected is less that 5%, thus assuring the ability of this signaling system to respond to the external perturbation, without negative consequences. Reasonably this specific behavior could offer an important evolutionary advantage in terms of adaptation.

In addition, the scale free topology allows the identification of the hubs, thus providing the evidence of the molecules that play a key role in signaling system. In particular we have found that the most

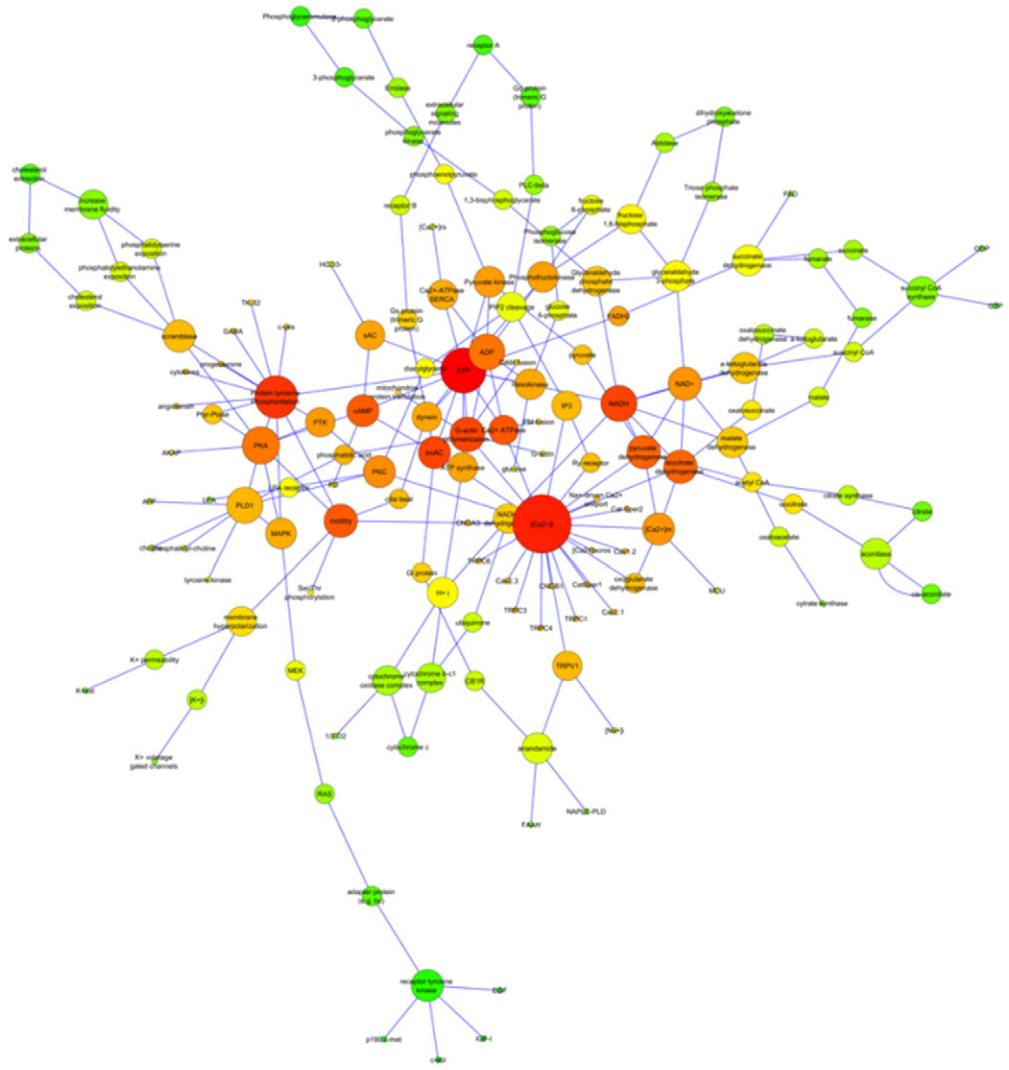

**Fig. 1.** Network representing human sperm activation.

connected nodes in our network were: the intracellular $Ca^{2+}$ concentration $[Ca^{2+}]_i$, ATP-ADP system, PKA, protein tyrosine phosphorylation, and $PLD_1$.

It is not surprising that $[Ca^{2+}]_i$ is the most connected node within the network: as it is well known, during capacitation $[Ca^{2+}]_i$ increases and in the absence of $Ca^{2+}$ this process does not take place [39,40]. The control of $[Ca^{2+}]_i$ is assured by different mechanisms. As first, the basal calcium clearance is controlled by the plasma membrane $Ca^{2+}$-ATPase, which exports a cytoplasmic $Ca^{2+}$ ion and imports one or two extracellular protons at the expense of ATP. When $[Ca^{2+}]_i$ is elevated, the plasma membrane $Na^+$–$Ca^{2+}$ exchanger operates in forward mode exporting an intracellular $Ca^{2+}$ ion and importing approximately three $Na^+$ ions [41,42]. Sarcoplasmic–endoplasmic reticulum $Ca^{2+}$-ATPase pumps and the mitochondrial $Ca^{2+}$ uniporter [43] operates in controlling the calcium flux from and to intracellular storages: acrosome or in mitochondria, respectively.

The increase in $[Ca^{2+}]_i$ occurring during capacitation is due to the influx of extracellular calcium through specific channels. CatSper channels are four pore-forming channel proteins (CatSper1–4) located on the sperm membrane. They contain six transmembrane-spanning (TM) domains, their overall sequences are similar to those of CaVs and they have the T/S-x-E/D-x-W signature sequence in the ion selectivity filter

**Table 1**
Main topological parameters of human sperm activation network.

| Parameter | Value | |
|---|---|---|
| N° nodes | 151 | |
| N° edges | 202 | |
| Clustering coefficient | 0.028 | |
| Diameter | 20 | |
| Averaged n° neighbors | 2.662 | |
| Char. path length | 6.546 | |
| Most connected nodes (n° of links) | $[Ca^{2+}]_i$ | (25) |
| | ATP | (14) |
| | Tyr-phosphorylation | (13) |
| | PKA | (9) |
| | ADP | (8) |
| | PLD1 | (8) |

**Table 2**
Result of power law fitting of IN and OUT of capacitation network.

| | Capacitation | |
|---|---|---|
| | In | Out |
| r | 0.988 | 0.997 |
| $R^2$ | 0.890 | 0.828 |
| b | −1.542 | −1.993 |

region. The intracellular NH2 terminus of CatSper1 is rich in histidines (49 out of 250 amino acids in mice), which may be related to its $pH_i$ sensitivity. The S4 segments of all CatSpers channels have charged residues (R/K) at every third position, a signature of voltage-gated ion channels [44]. Voltage-dependent calcium channels (VDCCs) are a group of voltage-gated cationic with a permeability to calcium about 1000-fold greater than to sodium. At resting membrane potential, VDCCs are normally closed, when they depolarize VDCCs open, thus allowing a fast elevation of $[Ca^{2+}]_i$ [45]. Transient receptor potential channels (TRP channels) are a family of 28 channels. They are divided into two groups: group 1 includes, TRPC ("C" for canonical), TRPV ("V" for vanilloid), TRPM ("M" for melastatin), TRPN and TRPA. Group 2 is constituted by TRPP ("P" for polycystic) and TRPML ("ML" for mucolipin). During capacitation it has been found that TRPV1 plays an important role in modulating of the $[Ca^{2+}]_i$ and the polymerization/depolymerization of actin [32]. Cyclic nucleotide-gated ion channels (CNG channels) are ion channels that activate in response to the binding of cyclic nucleotides cGMP and cAMP and either of a depolarization or a hyperpolarization event [46]. Their role during the acquisition of fertilizing ability of mammalian spermatozoa is still under debate.

The finding that $[Ca^{2+}]_i$ is the most connected node within sperm capacitation network, on the one hand, explains very elegantly why the management of $Ca^{2+}$ concentration is the most powerful tool to control the acquisition of fertilizing ability of male gametes, either inhibiting it (as is the case of calcium chelators present in extender used for cooled sperm conservation) or activating it (as is the case of high calcium concentration in buffers used in in vitro fertilization techniques). On the other hand, it straightens the concept that in spermatozoa $Ca^{2+}$ is not only a homeostatic factor and a second messenger, but, as it happens in excitable cells such as neurons, myocardiocytes, and muscular cells, it controls and modulates the crucial physiological events in cellular life, in our case the induction of AR.

The ATP–ADP system is the main energetic source of spermatozoa where the production of metabolic energy is guaranteed by the glycolysis exclusively, by mitochondrial oxidative phosphorylation exclusively, or by a combination of both pathways, depending on the species [47].

PKA is involved in several biochemical events, cross linking different pathways, in accordance with its role of superconnected node. It responds to the activation stimulus exerted by $Ca^{2+}$ and by $HCO_3^-$ which are transported within the sperm cell by cationic channels ($Ca^{2+}$) or by a $Na^+/HCO_3^-$ cotransporter ($HCO_3^-$). Both messengers concur in increasing cAMP, via a soluble adenylyl cyclase (sAC). Interestingly, this enzyme represents as an important player in capacitation biochemistry and functionally links the sperm cell physiology to the environmental bicarbonate concentration, thus allowing the information exchange with the female structures [48,49]. cAMP, in turn, acts activating the PKA activity. This enzyme modulates the activity of a myriad of other enzymes, either activating or inhibiting them, by transferring phosphate groups. The target proteins are involved in several biochemical processes involved in acquisition of the hyperactivated motility and the change in protein phosphorylation pattern appears to be a necessary prerequisite for reaching the ability to fertilize the oocyte, and has been demonstrated to increase either in flagellum or in sperm head [50–52]. The sophistication of control of PKA signaling during capacitation is demonstrated by the structural link of this enzyme with the A-kinase anchor proteins (AKAPs), which concur in the formation of organized functional domains within the sperm cytosol [53].

PLD1 plays a pivotal role in controlling the state actin polymerization. In particular MAP-kinase, tyrosine kinase, and ADP-ribosylation factors are involved in PLD activation, leading to phosphatidyl-choline hydrolysis to produce phosphatidic acid, which activates the polymerization of G-actin to F-actin [54–56].

Other highly linked nodes are those representing terminal events (such as protein phosphorylation or membrane fusion). Reasonably this is due to the redundancy of biochemical signaling, as a safety strategy to overlap partial failure of the system.

Interestingly, about 45% of nodes have exactly two links: one input and one output. This finding, together with the very low value of clustering coefficient, suggests that the network could have an organization that optimizes its signaling transduction-dedicated function.

Indeed, it exists a unidirectional flux of molecular messages from the beginning (input terminal) to the end (output terminal) of the chain, avoiding the presence of loop or clusters that could slow and interfere with the propagation of messages and, on the same time, assuring the specificity of signal transfer. In this context we can speculate that, once started, capacitation will proceed until it is completed, without multiple check-points or feedback loops. After all, spermatozoa are disposable cells: once activated they could have only two destinies to fertilize or to be lost.

Also the values of characteristic path length (about 6.5, see Table 1) are coherent with the signaling-devoted architecture of capacitation. Indeed, any molecule involved in capacitation interacts with any other through a small number of passages, thus the loss of information due to the signal decrease is minimized and, consequently, the signal efficiency is maximized. In addition, any local perturbation in signaling system could reach the whole network in a short time, thus increasing the system adaptation to intracellular and extracellular stimuli.

A further characteristic of capacitation network is that the activating signals are markedly most expressed than the inhibiting ones (about 95% vs. about 5%). This could be due to two different reasons:

- it is possible that the interest to recreate in vitro sperm capacitation in the contest of Assisted Reproductive Technologies leads the Researchers to study and to describe mainly the capacitation-promoting events. Thus the activating signals are not the most expressed but the most studied and, as a consequence, the most represented in scientific literature;
- the spermatozoa are functionally disposable cells. From a teleological point of view their fate is the completion of capacitation and, after all, the AR and the fertilization. Thus, it is possible that most of the biochemical pathways are objective-oriented leading sperm cells to complete capacitation.

From these findings it is possible to take some conclusion. As first, it is evident that the approach we adopted could be advantageously used to model and to study the capacitation, offering the possibility to infer biological information not otherwise obtainable. Indeed, it is possible to highlight the whole design of signaling cascades and to find a rational of the structural motifs that characterize the ensemble of molecular events involved in capacitation. In addition, it is possible to identify the most important molecules involved in that process, thus offering possible targets for *in vitro* protocol optimization/, diagnostic and therapeutic strategies development.

A further advantage of this approach is the possibility to make a comparative study by analyzing among the topology of networks representing the same process among different species. Indeed, the networks obtained from data referred to different organisms could have a different organization and topology. For instance, we have carried out the comparison of the biological network representing spermatozoa capacitation/activation among mammals, sea urchin and *Caenorhabditis elegans* [57].

Sea urchins are members of the *Phylum Echinodermata*, they are dioecious, and have five gonads. In this species the fertilization occurs in seawater [58]: sea urchin spermatozoa are released in saltwater, outside male organism, and became motile only once they contact with seawater. They swim up the oocyte, driven by chemoattractant molecules dispersed by the homologous oocytes the sperm-activating peptides (SAPs) [59]. When spermatozoa encounter the egg jelly, the exocytosis of the acrosomal vesicle occurs and the $pH_i$-dependent polymerization of actin leads to the extension of an acrosomal tubule, which exposes a new bindin-covered membrane which will fuse with the egg [60,61].

*C. elegans* is a 1 mm long worm, living in the ground. It has hermaphrodites (99.95% of individuals) and males (0.05% of individuals). Males

have a single-lobed gonad, a *vas deferens*, and a tail specialized for mating [62] while hermaphrodites have two ovaries, an oviduct, a chamber where oocytes are fertilized by sperm (the so called "spermatheca") and a single uterus. *C. elegans* spermatozoa are characterized by the absence of the flagellum and the lack of the acrosome, while they contain many membranous vesicles, the Membranous Organelles (MOs). They acquire their motility at the end of the process, the spermiogenesis or sperm activation, in which the spherical spermatid extends a pseudopod. This event requires an important reorganization of the cytoskeleton, constituted by a Major Sperm Protein (MSP) instead of actin, and of membrane microdomains [63].

As evident, the reproductive biology and ecology of human, sea urchin and *C. elegans* are completely different, thus comes the question of whether the organization of signal transduction is different in examined species or if may have common motifs. The use of biological networks based computational models could provide the answer to this question. Indeed, it was found that all three networks follow the same scale free topology and have a similar architecture. In addition also in the case of sea urchin and *C. elegans* the most connected node is $[Ca^{2+}]_i$. In sea urchin $Ca^{2+}$ enters the sperm cell through voltage dependent channels (Cav1.2 or 1.3) or through cAMP or cGMP (SpHCN1 or 2) gated channels and it is involved in the control of cAMP, cGMP and in the PIP2/IP3 pathways [45]. In *C. elegans* $[Ca^{2+}]_i$ is controlled by membrane channels and by sequestering the ion in intracellular stores and it concurs in modulating PIP2/IP3 signaling pathways and in regulating the MO fusion and the onset of motility [63]. The other hubs are molecules involved in energetic balance, such as ATP, or intracellular messengers such as $[H^+]_i$, which in sea urchin and *C. elegans* is a key element in control of many biochemical events (membrane polarization, the rearrangement of cytoskeleton proteins and the motility) [63,64], cAMP and cGMP. In particular, these two cyclic nucleotides concur in signal transduction by modulating the activity of several membrane channels, kinases, phosphatases and many other molecules.

These data demonstrates that it is possible to carry out inter-species comparison of species specific biological phenomena, obtaining reliable quantitative results that could be useful in comparative physiology.

### 3.2. Another example of cellular signaling complexity: The endocannabinoid system

The biological networks are extremely ductile tools, thus it is possible to use them also to explore the architecture of a signaling system active in different cells and in different anatomical and functional contexts. It is the case, for instance, of the endocannabinoid system (ECS), which is a widely expressed signaling system in human and mammalian organisms, where it is involved in a myriad of functions. ECS is constituted by lipidic ligands, their precursors and metabolites, their synthetic and hydrolyzing enzymes, their receptors, and by specific carriers. The two most studied endogenous ligands are the *N*-arachidonoylethanolamine (anandamide, AEA) and the 2-arachidonoylglycerol (2-AG), that bind type-1 and type-2 cannabinoid receptors ($CB_1$ and $CB_2$) [65], GPR55, a recently discovered putative "$CB_3$" receptor [66,67], and to nuclear receptors peroxisome proliferator-activated receptors (PPARs) [68]. AEA, in addition, binds the transient receptor potential vanilloid type 1 (TRPV1) channels [69]. AEA is synthesized by *N*-acylphosphatidylethanolamine-specific phospholipase D (NAPE-PLD), although alternative biosynthetic pathways have been described, including members of PLA/acyltransferase family like $Ca^{2+}$-independent N acyltransferases and multistep pathways via *N*-acylated lysophospholipids [70]. 2-AG synthesis occurs through a rapid hydrolysis of inositol phospholipids by a specific phospholipase C (PLC) to generate diacylglycerol (DAG), which is then converted into 2-AG by an sn-1-DAG lipase (DAGL) [71,72]. AEA is hydrolyzed by membrane-bound fatty acid amide hydrolase (FAAH), thus producing arachidonic acid (AA) and ethanolamine [73], whereas 2-AG is degraded to AA and glycerol by a specific mono-acylglycerol lipase (MAGL) [74]. The in/out trafficking of endocannabinoids is hypothesized to depend from the activity of specific endocannabinoid membrane transporter (EMT), whose existence and identity are still under debate [75].

To date, it has been found that ECS is actively involved in control of several biological processes of pivotal importance in physiological and pathological conditions, such as memory and learning, mood, fear, food intake, immune response, and reproduction, neuroinflammatory and neurodegenerative diseases, anxiety, depression, immune deficiency, obesity, skin disorders, and fertility/infertility [76–82].

The analysis of biological network representing the interaction among the molecules belonging to ECS allows one to study its architecture, taking into account its complexity, thus avoiding to reduce our attention to the single molecular components.

The data included in the ECS database are mainly referred to murine model (including knockout mice) and were considered only if confirmed by a large consensus and robust methods: they were considered only when they were supported my multiple experimental evidences (at least three independent studies), possibly obtained with different experimental approaches. Freely available and diffusible molecules such as $H_2O$, $CO_2$, $P_i$, $H^+$ and $O_2$ were omitted from the model, and in some cases the record represented complex events (e.g., cell adhesion or protein tyrosine phosphorylation) rather than a single molecule, because individual components of these ensembles are still unknown.

As first it was evident that, also in this case, the topology of ECS network is keeping with the BA model (the exponent of in and out node degree power law is, respectively, −2.188 and −1.078, see Table 4, and the clustering coefficient is 0.0009, see Table 3), thus suggesting some inference relevant to understanding the ECS biology. In particular it is characterized by robustness against random damage (as it was the case of capacitation), controllability and easy navigability (Fig. 2 and Tables 3 and 4).

The node size is proportional to the connection number and the node color gradient is dependent from the closeness centrality. This parameter is computed as: $C_c(n) = 1 / \text{avg}(L(n,m))$, were L (n,m) is the length of the shortest path between two nodes n and m. The closeness centrality of each node ranges from 0 (green) to 1 (red) and it is a measure of how fast the information spreads from a given node to the other nodes. The spatial network arrangement is obtained by using the Cytoscape Force-Directed Layout.

The number of nodes represents the total number of molecules involved; the number of edges represents the total number of interaction found; the clustering coefficient is calculated as $CI = 2nI / k(k-1)$,where nI is the number of links connecting the kI neighbors of node I to each other; the network diameter is the largest distance between two nodes; the Averaged n° neighbors represent the mean number of connection of each node, and the Char. path length gives the expected distance between two connected nodes.

**Table 3**
Main topological parameters of endocannabinoid system activation network.

| Parameter | Value | |
|---|---|---|
| N° nodes | 123 | |
| N° edges | 189 | |
| Clustering coefficient | 0.0009 | |
| Diameter | 12 | |
| Averaged n° neighbors | 3.073 | |
| Char. path length | 4.715 | |
| Most connected nodes (n° of links) | AEA | (45) |
| | 2-AG | (22) |
| | $[Ca^{2+}]_i$ | (12) |
| | $CB_1$ | (9) |
| | cAMP | (8) |
| | $G_s$-proteins | (8) |
| | $CB_2$ | (6) |
| | TNF-α | (6) |

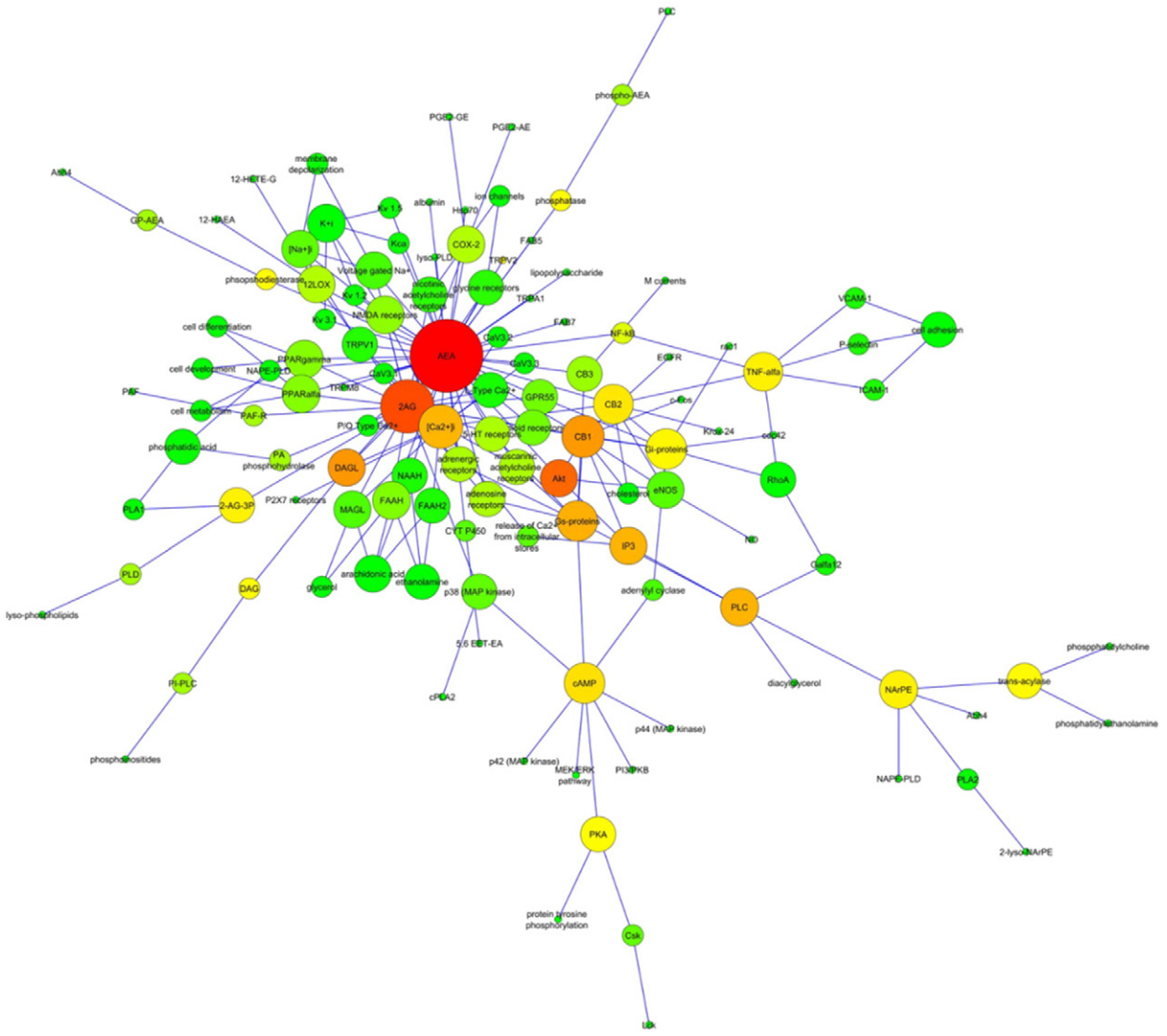


**Fig. 2.** Networks representing ECS.

These last two characteristics are of great relevance. Indeed, the small number of highly linked nodes implies that it will be possible to control and to modulate the whole system simply acting on a few molecules, thus minimizing the energetic cost and facilitating and accelerating the cell response. This strategy is very effective and, incidentally, offers the advantage of being more prone to external manipulations, for instance, through biotechnological or pharmacological interventions: the identification of a limited number of target molecules (the hubs) helpful in controlling the whole system could be exploited to develop diagnostic or therapeutic strategies. The easy navigability is due to the virtual absence of clustering and by the low values of the characteristic path length and of the averaged number of neighbors. In addition, the value of clustering coefficient near zero implies also the absence of redundancy within the network, indeed randomly picking two nodes into the network there is a high probability that only a single path between them exists. This could lead us to suggest that ECS is designed in a target-oriented manner, avoiding the diffusion of specific messages. Ultimately, this feature is keeping with the classical "small world" concept, that implies that ECS is able to elaborate and to transfer molecular messages from different cellular systems in a fast and specific way, strengthening the idea that it acts as a controller of integrated responses against homeostatic perturbation of the organism, as it has been documented within the central nervous system (CNS) or the immune system [83–85].

**Table 4**
Result of power law fitting of IN and OUT of endocannabinoid system network.

| | Endocannabinoid system | |
|---|---|---|
| | In | Out |
| r | 0.976 | 0.964 |
| $R^2$ | 0.9.15 | 0.684 |
| b | −2.188 | −1.078 |

The adoption of biological networks allowed also the exploration of different roles of ECS ligands in ECS network, thus supporting and completing some new experimental evidences. In particular it was found that AEA and 2-AG have a different number of links: 45 and 22 respectively. This suggests that AEA acts as a ubiquitous player at multiple target receptors within the ECS network, while 2-AG plays a role as CB1/CB2 agonist in more specialized contexts. In addition AEA and 2-AG are characterized by the higher values in betweenness centrality, thus suggesting that they exert a higher control on information flow through the ECS network, and act as a bottle neck in information transduction: all the messages to be transferred from the input terminal of the network to the output must pass through one or both of them.

Further, it was possible to identify the hubs involved in post-receptorial signal transduction: $[Ca^{2+}]_i$ and cAMP, both of them behave

as a transducer of ECS messages and integrate ECS with other signaling pathways, thus giving rise to a coordinate response to intra- and extracellular stimuli.

In addition, our results pointed out that ECS is a "membrane system", since about 75% of nodes behaving ECS network are directly located on the cell membrane, or exert their activity on this cell compartment. This is in line with very recently provided data about the role of membrane lipid composition as a key determinant in driving endocannabinoid signaling, and in particular with the finding that cholesterol controls the CB1 activity in membrane subdomains known as "lipid rafts" [86–90] and, as it happens in spermatozoa, cholesterol trafficking controls the AEA function and, in turn, the AEA function modulates membrane dynamics [19,30].

The use of the biological network-based approach also allows one to explore in detail the functional role exerted by molecules involved in ECS physiology by assessing the effect of the removal of correspondent nodes from the network, as it is the case of AEA and 2-AG, arachidonic acid and FAAH.

In the first case, network integrity was completely destroyed by the removal of AEA and 2-AG: the connected component collapse to give rise to sparse connected components (Table 5). On the contrary, when the node representing the arachidonic acid was removed from the ECS network, its topology did not significantly change (Table 5). This in silico datum straightens and clarifies the experimental evidence that the biological activity of endocannabinoids is distinct from that of this fatty acid [91,92]. Analogously, the removal of node representing FAAH, that controls the endogenous tone of AEA in vivo and also cleaves 2-AG, did not significantly affect ECS topology (Table 5). Interestingly, FAAH is not a hub of ECS networks but it seems to exert a strong control on network function by directly modulating the concentration of AEA, which is the most connected node.

This study represents a first step in modelization of ECS, in the future it will be interesting to extend the researches to the analysis of difference in the expression of ECS in different organisms, cells and tissues, as well in different moments of the life of cells.

## 4. Some common aspects of sperm capacitation and ECS networks

The adoption of this computational approach leads us to achieve important results. As first, it makes available an instrument useful to identify the molecules (or the group of molecules) playing key roles in cell signaling, and the possible consequences of their manipulation. Reasonably, these molecules will be the best candidates to develop diagnostic tools as well as to be selected as target for therapies. In this regard it is interesting to note that, on the one hand, the availability of molecular tools (such as specific inhibitors) and of animal models (particularly KO mice) could justify the research effort on a specific topic and, as a consequence, the amount of available information. On the other hand, we are confident that the number of publications on each topic should only have a limited influence on the number of links per node. Indeed, in a recent, independent study we have assessed the correlation of the number of articles on different topics (i.e., the smooth and striated muscle contraction, the neurotransmitter release cycle of six neurotransmitters [norepinephrine, acetylcholine, γ-aminobutyric acid (GABA), serotonin, glutamate and dopamine], the visual phototransduction (rods), the sperm capacitation, the insulin signaling pathway, the p53 pathway, the regulation of retinoblastoma protein (pRb), the mitochondrial ATP metabolism, the glucose metabolism, the signaling events mediated by stem cell factor receptor c-Kit (CD117), and the circadian clock) with the topological parameters of corresponding networks and we have found no statistically significant correlation [93].

In addition, in both cases (i.e. sperm capacitation and endocannabinoid system) the use of networks as models allows one to study in detail the flow of information within the system. In particular, considering the network as directed, it is possible to identify the input and output terminals of signaling cascade, by observing the nodes with only one link. In the classical BA model of scale free networks, these nodes being the less connected ones, are also the less important ones. For instance in the WWW and internet the sites with only one link are considered destined to disappear [7]. Instead, in our case, the nodes with one link are those representing the terminal events of signaling or are the input terminal connecting intracellular biochemistry with the surrounding milieu.

Ultimately, this approach offers the evidence that the study of biological systems necessarily must take into account the complexity of biological processes. It is not correct to refer to the ECS molecules as separated entities, independent of each other, and it is incorrect to search the determinant of male gametes fertilizing ability at molecular level: the fertility is an emergent propriety of the spermatozoon as a whole. Thus, all the researchers involved in the study of ECS activities, in sperm capacitation or more general in the study of cell signaling with the aim to develop diagnostic tools, drugs, new therapies, etc. in our opinion, will have great advantages adopting such systems biology approach. Indeed recently, further analysis, showed that the same architecture is shared also by several other networks representing signaling transduction pathways of relevant importance in mammalian biology [93]. This leads to conclude that signal cascades are characterized by important biological features such as robustness against random failure, specificity and efficiency in signal transmission.

**Table 5**
Main topological parameters of ECS, ECS without AEA and 2-AG nodes, and ECS without arachidonic acid node. See text for details.

| Topological parameter | ECS minus AEA and 2-AG | ECS minus arachidonic acid | ECS minus FAAH |
|---|---|---|---|
| N° nodes | 121 | 122 | 122 |
| N° edges | 120 | 185 | 184 |
| Connected components | 19 | 1 | 1 |
| Diameter | 8 | 12 | 12 |
| Clustering coefficient | 0.0130 | 0.0009 | 0.0009 |
| Averaged n° neighbors | 1.983 | 3.033 | 3.016 |
| Characteristic path length | 3.014 | 4.723 | 4.748 |
| *IN* | | | |
| γ | −1.928 | −2.191 | −2.190 |
| r | 0.999 | 0.976 | 0.980 |
| $R^2$ | 0.917 | 0.903 | 0.904 |
| *OUT* | | | |
| γ | −2.084 | −1.059 | −1.082 |
| r | 0.999 | 0.953 | 0.963 |
| $R^2$ | 0.985 | 0.684 | 0.691 |

AEA = arachidonoylethanolamide; 2-AG = 2-arachidonoylglycerol.

## 5. Conclusions

Biological networks are flexible and reliable tools to explore the cell signaling pathways either for studying a complex function (as it is the case of sperm capacitation or activation) or for describe a complex galaxy of signaling molecules (as ECS) expressed in different tissues and organs. The advantages are constituted by the possibility to model complex events taking into account the molecules involved as well as their interaction, even in the case of qualitative information. In addition, which is the most important, it is possible to use the biological networks to build models based on data from high throughput technologies, thus making possible to manage myriads of data by mathematical models (the so called "big data problem"). The statistical analysis of network topology becomes the instrument to infer important data, related to the biological meaning of the event of interest, expressed quantitatively. This is very important: it is on the basis of the possibility to perform comparisons among different cells, tissues, organisms, etc., to study the chronological evolution of events, to evaluate the effect of experimental manipulation of the system.

At present, the application of network science to biology is an amazingly growing field, which dynamically evolves toward new challenges, such as the investigation of new proprieties of biological networks, looking at the network dynamics and at the development of local motifs as specific signature of biological events [94–96].

Intriguingly, the use of such computational models could improve the knowledge and the understanding of biological systems, thus allowing to take great advantage by the adoption of high throughput technology. At the same time the continuously growing number of molecular data will furnish to computational biologists the opportunity to refine the models, thus giving rise to a virtuous circle.

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