Neuronal signaling repertoire in the mammalian sperm functionality

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Abstract
The common embryonic origin has been a recurrent explanation to understand the presence of “neural receptors” in sperm. However, this designation has conditioned a bias marked by the classical neurotransmission model, dismissing the possibility that neurotransmitters can play specific roles in the sperm function by themselves. For instance, the launching of acrosome reaction, a fundamental sperm function, includes several steps that recall the process of presynaptic secretion. Unlike of postsynaptic neuron, whose activation is mediated by molecular interaction between neurotransmitter and postsynaptic receptors, the oocyte activation is not mediated by receptors, but by cytosolic translocation of sperm phospholipase (PLCζ). Thus, the sperm has a cellular design to access and activate the oocyte and restore the ploidy of the species by an “allogenic pronuclear fusion.” At subcellular level, the events controlling sperm function, particularly the capacitation process, are activated by chemical signals that trigger ion fluxes, sterol oxidation, synthesis of cyclic adenosine monophosphate, protein kinase A activation, tyrosine phosphorylations and calcium signaling, which correspond to second messengers similar to those associated with exocytosis and growth cone guidance in neurons. Classically, the sperm function associated with neural signals has been analyzed as a unidimensional approach (single ligand-receptor effect). However, the in vivo sperm are exposed to multidimensional signaling context, for example, the GABAergic, monoaminergic, purinergic, cholinergic, and melatoninergic, to name a few. The aim of this review is to present an overview of sperm functionality associated with “neuronal signaling” and possible cellular and molecular mechanisms involved in their regulation.

Summary Sentence
The mammalian sperm function is regulated by signals that are present in the male and female reproductive tract. Several of this signals correspond to neurotransmitters that activate the transduction signaling implicated in molecular control of sperm physiology.
Introduction

Sperm cell generation

Numerous studies have added to the understanding of mechanisms underlying sperm formation. Over the last decade, a research has broadened our knowledge of the genes critical for spermatogonial stem cells’ (SSC) self-renewal and proliferation. The establishment of the human male germ cell lineage starts during embryogenesis at 4 weeks of gestation [1]. Studies on mice have shown that primordial germ cells (PGC) originate from the proximal epiblast in the yolk sac in response to bone morphogenetic protein stimulation from the nearby visceral endoderm and extraembryonic ectoderm [2]. From this start point, germ cell lineage is initiated by the specification of embryonic cells to adopt the germ cell fate under the control of bone morphogenetic protein 4 (BMP4) and BMP8b, starting with the formation of PGC precursors expressing PRDM1 (BLIMP1) and PRDM14 [3]. These PGC precursors are detected around embryonic day (E) 6.25 in the proximal epiblast, which further evolves to alkaline phosphatase and Stella-expressing PGCs at E7.25 [12]. At E7.5, these sexually undifferentiated PGCs start migrating toward the genital ridge until they reached gonads. In males, PGCs become resident and enclosed by Sertoli cells in gonads, forming testicular cords at E12.5dpc in rodents [4]. Recent reports indicate that PGCs migrate very little, and their migration is aided by hindgut expansion and growth movements [5,6]. Furthermore, it has been proposed that human PGC move in a concomitant manner with the simultaneous growth of autonomic nerve fibers from the dorsal mesentery until the developing gonad [7,8]. In this sense, the enteric nervous system of the mid- and hindgut expresses KIT ligand (KITLG) [9]. The expression of KITLG by the gut autonomic nerve fibers is important, since the interaction of KIT-KITLG has shown to be instrumental in the modulation of migration and survival of PGC into the embryo [9–11]. However, although the relationship of enteric nervous fibers and PGC seems clear, further studies are needed to clarify the exact process of PGC migration. In any case, PGCs undergo genomewide DNA demethylation during the migration phase, thus leading to the elimination of parental imprints [12,13]. Sex determination is established at E12.5 in both germ and supporting somatic cells, based in part on the expression of the Sry gene in male somatic cells [14]. Once the testicular cords have formed, germ cells present in the cord are referred to as gonocytes [15,16–18]. The decision of gonocytes to enter the female or male germ cell pathway is influenced by the somatic compartment surrounding the germ cells. In males, mesenchymal, Sertoli, and Leydig cells are crucial for the formation of the male gonadal paracrine and endocrine interactions. At E7.5, these sexually undifferentiated PGCs start migrating toward the genital ridge until they reached gonads. In males, PGCs become resident and enclosed by Sertoli cells in gonads, forming testicular cords at E12.5dpc in rodents [4]. Recent reports indicate that PGCs migrate very little, and their migration is aided by hindgut expansion and growth movements [5,6]. Furthermore, it has been proposed that human PGC move in a concomitant manner with the simultaneous growth of autonomic nerve fibers from the dorsal mesentery until the developing gonad [7,8]. In this sense, the enteric nervous system of the mid- and hindgut expresses KIT ligand (KITLG) [9]. 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In males, mesenchymal, Sertoli, and Leydig cells are crucial for the formation of the male gonadal paracrine and endocrine interactions (e.g. inhibin B and testosterone) [19,20]. The initiation of the male path of sexual development is dependent on the activation of several genes such as DHH, FGF9, CBX2, DMRT1, AMH, SRY, and SOX9 [21,22]. The fate of male germ cells during gonads colonization follows a two-phase chronological process. The first phase takes place from embryonic age to infancy. It consists of successive multiplication phases of PGCs and subsequent gonocytes leading to the formation of the first spermatoogonia, including SSCs [15]. The second phase, starting at puberty and extending throughout male adult life, comprises spermatogonial waves that are initiated by the entry of spermatogonial waves into meiosis and further spermiogenesis to form immature sperm [23]. In summary, normal spermatogenesis is a complex growth and maturation process encompassing three distinct stages: (1) mitotic proliferation and maturation of spermatogonia; (2) meiotic cleavage to form haploid cells, and (3) final maturation into elongated spermatids and spermatozoa (spermiogenesis).

Sperm capacitation, gametic interaction, and acrosome reaction

After spermiogenesis and epididymal maturation, mature sperm are stored in the final end of epididymis until either ejaculation or their ultimate death and reabsorption if they are not ejaculated. However, mature epididymal sperm have not the ability to fertilize an oocyte. In fact, mammalian spermatozoa need to suffer a complex series of functional changes after ejaculation in order to achieve fertilizing ability [24,25]. The process by which sperm underwent the whole array of specific functional changes associated with the fertility success was subsequently called capacitation [24,25].

Sperm capacitation is a complex and finely tuned process in which membranous, ionic, and metabolic alterations occur at specific times and locations and in which several components of signal transduction pathways are involved [26,27]. Under a biochemical point of view, the capacitation process includes an efflux of cholesterol from the plasma membrane leading to an increase in membrane fluidity and permeability to bicarbonate (HCO₃⁻) and calcium ions. In this context, several changes happen, highlighting increases in pH, intracellular Ca²⁺, cyclic adenosine monophosphate (cAMP) levels, hyperpolarization of the plasma membrane [28], and time-dependent changes in protein tyrosine phosphorylation, phenomena that are downstream of the activation of protein kinase A (PKA) [29]. From a chronological point of view, capacitation is divided into two groups of signaling events: fast events and slow events [30]. The fast events start immediately after ejaculation through the simultaneous action of several extracellular factors present in both the seminal plasma and the female genital tract. In summary, the most relevant feature linked to the launching of the fast events of capacitation is the activation of the cAMP-dependent PKA through pathways involving bicarbonate activation of cAMP synthesis and the downregulation of serine-threonine phosphatases through the action of kinases from the Src family [31,32]. At this respect, the Ca²⁺/HCO₃⁻-modulated PKA activation seems to be mediated through the uptake of both modulators via both the sperm-specific Ca²⁺ channel (CatSper) and the Na⁺/HCO₃⁻ cotransporter, respectively [30]. The PKA activation is concomitant with a synchronous activation of sperm motion, which is characterized by vigorous and asymmetric flagella movements [33]. The fast events of sperm capacitation are concomitant with the first steps of the sperm progression through the female genital tract, until the remnants spermatozoa reached oviduct. The launching of the fast events of capacitation is followed, in a more or less synchronous manner, by the start of the slow events, which will reach the completion once sperm reach oviduct. The slow events of capacitation include changes in the pattern of sperm movement that become evident once sperm are detached from oviduct following chemiotactic signals linked with the launching of ovulation [34,35]. The specific sperm motion pattern linked with the establishment of the slow events of capacitation is named hyperactivated motility, which is characterized by nonlinear trajectories and wide amplitude of both head and tail sperm beating [34,35]. The beginning of the slow events of capacitation is marked by the removal of cholesterol from the membrane, thus increasing sperm membrane fluidity [30]. Concomitantly, other prominent phenomenon observed during the establishment of the slow events of capacitation is an overall increase in cell protein tyrosine phosphorylation.
phosphorylation [36,37]. This increase was in fact started following the early activation of PKA induced as a relevant feature of the fast events of capacitation. The activation of the sperm PKA will subsequently launch a complex activation cascade of a wide array of separate protein kinases, which ultimately will cause the observed increase in protein tyrosine phosphorylation observed in the slow events of capacitation. Finally, another important feature observed during the achievement of capacitation is an important change in the structure of the sperm cell surface, which is initiated during the rapid events phase and is ultimately developed after the contact of sperm with oviductal cells [38,39]. Thus, in the oviduct, the epithelium adsorbs proteins from the sperm surface and also secretes glycoproteins that would play a role in the launching of the acrosome reaction [26]. A delicate reorientation and modification of sperm surface molecules takes place when sperm are activated by capacitation factors. These surface changes are probably required to enable the sperm to bind to the extracellular matrix of the oocyte on the zona pellucida (ZP) [40]. Thus, it is well known that a specific set of sperm surface proteins located at the equatorial region is involved in adhesion and subsequent fusion of both gametes during the first steps of the fertilization process [41]. As a result, the union of sperm to oocyte surrounding structures through specific proteins majoritary located at the equatorial region is a mandatory requirement before the launching of the sperm acrosome reaction as the following step in the process of sperm penetration into the oocyte. In summary, the ultimate goal of all changes linked with the achievement of the capacitation status will be to yield sperm with full ability for carrying out the acrosome reaction after stimulation with the appropriate inducers. In this manner, the final, capacitated sperm will be a cell that will show the ability to display an hyperactivated motility, a strong chemotactic behavior in front specific factors [42] and finally the ability to fertilize an oocyte [31].

The launch of the acrosome reaction in previously capacitated sperm is subjected to a series of specific conditions. The acrosome reaction is relatively easy to detect on live sperm due to the extraordinary size of the membrane surface area that is subject of the acrosome exocytosis phenomenon [43]. In this way, the study of posttranslational changes of acrosome proteins linked to the launching of the acrosome reaction is relatively easy. Taking this into consideration, the molecular architecture of the sperm surface and the interaction of sperm surface proteins with structures surrounding the oocyte like the ZP will be key aspects in order to understand the launching mechanisms of the acrosome reaction. At this respect, it is noteworthy that for over 30 years one of the central dogmas of the fertilization process in mammals has been that capacitated, acrosome-intact sperm requires the binding to the ZP before launching the acrosomal exocytosis [44,45]. However, 10 years ago Baibakov et al. by using a transgenic mouse model showed that sperm binding to ZP by itself is not sufficient to induce acrosomal exocytosis [46]. This would be the explanation regarding the requirement in the concomitant action of other effectors, such as progesterone, in order to launch the acrosome reaction [47,48].

Taking sperm capacitation and subsequent acrosome reaction as a whole, all of above-described steps are characterized by a specific spatial organization of sperm membrane molecules linked with phenomena like changes in the phosphorylation of specific proteins and in the exact composition of the sperm membrane. All of these changes will ultimately yield the observed changes in motion parameters, as well as they lead sperm to a successful interaction with the oocyte. This communication serves as a short overview of the molecular events occurring on the sperm surface, without providing exhaustive lists of protein candidates for each process.

Similar gene expression pattern between brain and testis

In silico analysis at the gene expression level of 17 human tissues indicates a similar gene expression pattern between human brain and testis [49], which identified genes whose expressions are specific to brain or testis, or shared by both tissues, respectively. Previously, there is evidence for supporting the notion that human testis and brain share some particular correlation and that could be implicated in speciation process [50]. Similarly, Graves proposed that genes in the SOX family involved in brain and testis determination share the same genetic origin [51]. Kitamura et al. found that mutation of Arx could lead to abnormal development of both the forebrain and testes in mice [52]. To date, many genes have been identified that show similar expression patterns in the brain and testis, which add to higher-level functional/structural similarities reported between testis and brain [50,53–59].

Neural signaling in mammalian sperm

Chemical signals are the universal language for communication between cells from bacteria and protozoa to somatic cells and gametes in invertebrates and mammals [60,61]. Neurons activate target cells by release of neurotransmitters present in vesicular secretions of the presynaptic neuron. This release of chemical signals in the synaptic space is essential for neuronal function with the neurotransmitters interacting with postsynaptic excitatory and/or inhibitory receptors to initiate, facilitate, or inhibit neurotransmission [62].

Sperm are swimming machines designed to find and inject genetic information into unfertilized eggs [61,63,64]. Sperm contain a single secretory granule (acrosome) in the anterior region of the sperm head, a nucleus with a haploid complement of condensed chromosomes, two centrioles, mitochondria, and a flagellum [60].

Acrosomal exocytosis (AR) at the egg’s surface is required for gamete fusion and egg activation during fertilization. The species-specific ligand that stimulates AR in sea urchins is a homopolymmer of sulfated polysaccharide in the egg’s jelly coat [65]. In mammalian eggs, the AR-activating point is an O-linked carbohydrate on zona pellucida glycoprotein 3 (ZP3) [66]. Binding of these chemical signals or agonists with their receptors on invertebrate and mammalian sperm triggers signal cascades involving ion fluxes, synthesis of cAMP, and other second messengers similar to those associated with exocytosis in neurons [61–64,67].

Invertebrate and mammalian sperm express receptors for many neurotransmitters and neuromodulators, e.g., acetylcholine (nicotinic and muscarinic types), adenosine and ATP [61], γ-aminobutyric acid (GABA) [68–70], serotonin [71], norepinephrine [72,73], and dopamine [58,74].

Sperm also express receptors for psychoactive drugs, e.g., nicotine [61,75,76], cocaine [61], opioids [77–79], cannabinoids [61,80–84], and melatonin [85,86].

Receptors for these neuroactive agents modulate normal sperm functions essential for fertilization, including respiration, motility, chemotaxis, capacitation, and AR [82]. Moreover, the sperm cell also has presence of several neurotransmitter transporters, e.g., GABA transporter [68], dopamine, norepinephrine, and serotonin transporters [59,71,74].

Specific neural signaling in sperm cells

Neurotrophins signaling

Neurotrophins are a family of proteins that regulate development and maintenance of sympathetic and sensory peripheral neurons, as
well as some cholinergic neurons [87]. The neurotrophins family includes nerve growth factor (NGF), brain-derived neurotrophic factor, neurotrophin 3 (NT-3), neurotrophin-4 (NT-4), and neurotrophin 5 (NT-5) [88]. The functions of neurotrophins are mainly mediated for tyrosine kinase (Trk) receptors. The binding of neurotrophins to Trk receptors results in receptor dimerization, kinase activation, and signaling cascades by creating docking sites for adaptor cytosolic proteins. These proteins couple these receptors to pathways such as the Ras/extracellular signal-regulated protein kinase (ERK) and mitogen-activated protein kinase (MAPK)-related pathways [89].

The main role of neurotrophins seems to be linked with a central trophic effect in the nervous system. However, this seems not to be the only role of neurotrophins, since they can be found at high levels in tissues other than nervous system. Thus, high concentrations of NGF have been reported to exist in peripheral tissues such as testsis [90,91] and ovary [92,93]. In addition, several observations linking NGF to sperm development have been reported. In this way, high levels of mRNA coding for NGF have been found in the lower mouse caput epididymis and corpus epididymis, suggesting a role for NGF in sperm maturation [90]. Moreover, high levels of NGF have also been detected in seminal plasma of several mammalians, and interestingly, this has been linked with the control of the ovulation process [94].

At physiological level, Jin et al. showed the presence of Trk receptors in hamster sperm and the stimulating effect of NGF on both sperm motility parameters and acrosome reaction in a time- and dose-dependent manner [95]. Moreover, the effect of NGF on the acrosome reaction was neutralized with the use of specific MAPK inhibitors [95]. In the same year, another group showed the presence of NGF and Trk receptors in ejaculated bovine sperm, reporting that NGF increases leptin secretion and improves viability at 20 μg/L without affecting both calcium levels and acrosome reaction. This suggests that the effect of NGF could be species specific [96]. More recently, Shi et al. confirmed by computer assisted sperm analysis system that NGF at concentrations higher than 1 μM in human sperm significantly increased several parameters of motility, highlighting the average path velocity, straight line velocity, curvilinear velocity, beat-cross frequency, and linearity [97]. In summary, the physiological significance of NGF in mammalian semen is still poorly understood. Despite this, NGF has evident effects in mammalian reproductive functions such as an ovulation inducing factor [94], motility activator [95,97], and sperm chemotractant effect on leukocytes [97]. This indicates that much more work is needed to fully elucidate the role of NGF as reproductive function modulator.

GABAergic signaling

The presence of a gabaergic system in sperm has recently been reported. Ionotropic GABAAR receptors (GABAAR), metabotropic GABABR1 and GABABR2 receptors, and GABA transporter 1 (GAT1) are present in sperm cells [68,98–100] where activation of GABAAR and GABABR alters the rate of acrosomal reaction [101]. GABAARs have been detected in pig, ram, and human sperm membrane preparations by radioligand-binding experiments [69,70]. Immunofluorescence and western blot studies with an antibody against the neuronal GABAARα1 subunit [102,103] demonstrated the presence of that subunit in ejaculated human sperm. The GABAARα1 subunit was localized primarily to the plasma membrane overlying the equatorial segment of motile or fixed permeabilized capacitated and noncapacitated ejaculated human sperm [103].

It is noteworthy that GABA itself is capable to induce the “in vitro” AR initiation in several mammalian species [101,103–106]. However, it should be noted that while progesterone and these same neurosteroid progesterone metabolites can increase neuronal GABAAR activation by GABA, the progesterone metabolites but not progesterone activate GABAARs in the absence of GABA [107]. The enzyme needed for GABA synthesis is present in the midpiece of ejaculated human sperm [108], but only very low levels of GABA or none have been reported in ejaculated human sperm [70,103]. GABA is also present in the mammalian oviduct (though its oviductal fluid concentration is not known) and can be secreted by that organ in vitro [109,110]. Since progesterone is still secreted by the cumulus of ovulated eggs [111], it could be involved in the activation of sperm GABAAR in vivo.

More recently, Vidal et al. found that Marlin-1, a GABAB receptor-binding protein is expressed in rat testis [112]. Additionally, they reported that Marlin-1 associates with GABAB-R1 receptors in testis even though the expression levels are lower than in the brain. These findings indicate that the presence of GABAB receptors type in testis extends to their interacting proteins that modulate their function in neurons. Thus, they argue in favor of conserved functional neurotransmitter receptor complexes in sperm. However, the specific role of Marlin-1 with or without association with GABA B in viability, motility, acrosomal reaction, or fertilization remains unknown.

Glycine signaling

Glycine receptors (GlyRs) are involved in the inhibition of central nervous system neurotransmission but have also been found in macrophages, leukocytes, and adrenal chromaffin cells [113,114]. Immunofluorescence studies demonstrated that GlyRs are present in the periacrosomal plasma membrane of ejaculated pig sperm (both capacitated and noncapacitated) and postfixed, noncapacitated and capacitated epididymal mouse sperm. Furthermore, western blot studies detected both a and b GlyR subunits in pig sperm extracts [115,116]. Although the neuronal GlyR is usually involved in inhibitory neurotransmission via Cl− influx and subsequent hyperpolarization, glycine binding to presynaptic GlyRs causes a “weakly depolarizing Ca2+ current,” presumably Cl− efflux, resulting in activation of Ca2+ channels and increased vesicular secretion [117]. A similar mechanism involving Cl− efflux may be involved in the ZP-initiated AR since sperm membrane depolarization and subsequent activation of voltage-activated Ca2+ channels are important steps for that event [118]. Moreover, based on the fact that strychnine is a classic GlyR antagonist at nanomolar levels [119], several in vitro studies with strychnine suggest a role for Cl− flux (presumably efflux as discussed above) via the GlyR in the ZP-initiated AR. Thus, at nanomolar levels, strychnine inhibited pig and mouse AR initiated by solubilized ZP, glycine-initiated AR of pig, human, hamster and mouse sperm, and human AR initiated by recombinant human ZP3 (rhZP3) [116,120–123].

Cholinergic signaling

Radioligand binding studies detected nicotinic acetylcholine receptors (nAChRs) in intact ram and rabbit sperm [124,125]. These nAChRs are ligand-gated, plasma membrane cation channels involved in fast synaptic transmission in the central and peripheral nervous systems and in muscle [126,127]. The nAChRs also have an important function in sperm, since the three most potent antagonists of the nAChRs, namely α-bungarotoxin, α-conotoxin IMI,
and methyllaconitine, completely blocked initiation of the AR by rhZP3 and solubilized mouse ZP in ejaculated human sperm and epididymal mouse sperm [123]. However, any of the described nAChRs antagonists were not able to inhibit human and mouse sperm AR initiation by both progesterone and the calcium ionophore A23187 [122]. Both the sperm nAChR and ChAT are localized to the postacrosomal region and tail in human sperm [128,129]. In sperm, ChAT might be activated upon ZP interaction with sperm leading to phosphorylation of the enzyme. Neuronal ChAT can be activated by PKC [130]. Carnitine acetyltransferase, another enzyme capable of ACh synthesis, is also present in mammalian sperm [131] and may be more important than ChAT for sperm acetylcholine synthesis [132]. An autocrine/paracrine ACh system including sperm ChAT, acetylcholinesterase, and nAChR was suggested previously (based on agonist and antagonist studies), as a means of motility control in sperm [76,133].

Opioids signaling

There are three basic types of opioid receptors designated: μ, δ, and κ. Each of these appears to be involved in the regulation of various aspects of reproduction [78]. The δ-opioid receptors have been found in human sperm [78], where their putative involvement in the modulation of sperm motility has been hypothesized. Likewise, all of three opioid receptors have been detected in fish sperm [79], whereas the presence of the μ-opioid receptor has been demonstrated in equine sperm cells in the head region, with greater intensity in the equatorial region postacrosome and part of the midpiece and tail [78]. Additionally, it has been demonstrated that sperm cells express μ-opioid receptors and κ-opioid receptors [78]. The latter result could be factored in the fact that the human semen contains the endogenous opioid peptide β-endorphin [134]. Despite these results, the effects of opioids on sperm are not yet completely understood. Thus, it is likely that the opioid system participates in sperm motility control through an inhibitory effect [78]. Notwithstanding, the exact mechanism by which opioids can exert this inhibitory effect is not known. In this context, one might recall that opioids affect cAMP production through inhibition of the adenylate cyclase activity in a similar manner than that of other sedative drugs such as the α-2 adrenergic agonist xylazine [135]. Moreover, the addition of the anesthetic drug ketamine antagonizes the effect of μ-opioid receptors, as does naloxone; interestingly, both drug acts through a blockage of the N-methyl-D-aspartate (NMDA) receptors [136].

On the other hand, Peterson and Freund compared the inhibitory effects of certain drugs on human sperm motility, including lidocaine, a local anesthetic [137]. This study showed that concentrations of 12 nM are sufficient to inhibit sperm motility. Taking into account that the cAMP-dependent PKA activity is instrumental in the maintenance of mammalian sperm motility, one could hypothesize that the inhibitory effect of opioids on sperm motility could be mediated by the inhibition of cAMP synthesis, probably by system involving NMDA receptors. Notwithstanding, further investigations will be needed in order to elucidate this point.

Cannabinoid signaling

The first studies that provided evidence that cannabinoids like both Δ9-tetrahydrocannabinol (THC) and anandamide (AEA) can directly affect the fertilizing capacity utilized sea urchin sperm as a model [138–140]. Interestingly, both THC and AEA act specifically on sperm and not the ovum [138]. The effects of both AEA and Δ9-THC on sea urchin fertilization were an enhancement of sperm motility that was concomitant with a potent blockage of the acrosome reaction [81,139,141,142]. Further studies demonstrated that the inhibitory effects of cannabinoid agonists on acrosome reaction are reversible and are mediated by processes of signal transduction involving the opening of Ca2+ and K+ channels, phospholipase A2 activation, etc., similarly to that observed in the regulation of neuronal synaptic secretion [65,123,125,126]. Interestingly, it has been reported that the sea urchin oocyte has the ability to produce AEA during fertilization [143]. In this context, it has been suggested that the acrosome releases AEA after being activated by the sperm in order to inhibit the acrosome reaction of other sperm than that penetrating the oocyte, thus preventing polyspermy [81,142].

Similar results to those observed in sea urchin have been observed in mammals. In this sense, it has been shown by radio binding assay that human sperm bind [3H]CP55,940 (Kd 9.71 ± 1.04 nM), a potent cannabinoid agonist, in a similar manner to urchin sperm [144]. Furthermore, methanandamide (AM-356), a nonmetabolizable synthetic cannabinoid, exerts a biphasic effect on mammalian sperm hyperactive motility, enhancing it at low doses (0.25 nM), and inhibiting it at high doses (2.5 nM). On other hand, the use of AM-356 in human sperm hemizona binding assay decreased their index at 50% [144].

Specific binding assays with cannabinoid agonists on human sperm suggested the presence of the cannabinoid receptor type 1 (CB1 or CNR1). Molecular analysis by RT-PCR and immunocytochemistry showed that human sperm express CB1, and activation exerts a negative effect in sperm motility and acrosome reaction. In addition, AEA does not alter the Ca2+ flow, whereas it induces a rapid hyperpolarization of the plasma membrane and a reduction in mitochondrial activity [83].

Very interesting genetic evidence has been reported in relation to the role of CB1 receptor on sperm motility. Thus, Ricci et al reported a significant reduction of “wild-type” sperm motility in the caput region compared to the tail region of the epididymis; however, the sperm of CB1-deficient mice from cauda region showed a strong increase of motility [145]. Cobellis et al. reported that the genetic and pharmacological inactivation of CB1 increases the number of motile sperm in mouse epididymal caput [146]. Likewise, blockers of endocannabinoid cellular uptake inhibit the potential sperm motility and alter the 2-arachidonoyl-glycerol (2-AG) gradient throughout the epididymis.

The existence of a complex endocannabinoid system with a putative modulatory role in capacitation and further acrosome reaction has been demonstrated in boar sperm [84]. Specifically, these authors disclose that the transient potential vanilloid 1 receptor (TRPV1) receptor has a role in stabilizing the sperm plasma membrane during capacitation, thus reducing both the premature and the spontaneous acrosome reaction. Meanwhile, a decrease of boar sperm fatty acid amide hydrolase (FAAH) activity would be necessary to achieve capacitation. A similar decrease on binding activity of both the AEA transporter and CB1 is linked to the achievement of boar sperm capacitation [84]. However, these are not the only molecules related with endocannabinoids metabolism that have been detected in sperm. Thus, recently the TRPV1 has been also found in sperm cells [147]. In fact, results of these authors suggest that both CB1 and TRPV1 would play key roles in the achievement of sperm capacitation. Specifically, the CB1 is implicated in the G protein/cAMP/PKA pathway in early stages of post ejaculation, thus promoting the maintenance of membrane stability and avoiding
premature acrosome reaction. The TRPV1, on the contrary, is activated in the latest stages of capacitation, and allows the rapid increase in intracellular calcium concentration needed for membrane fusion and acrosome reaction [147,148].

The important role of endocannabinoids in the modulation of the overall sperm function has been observed in other species. Thus, mouse sperm have a fully functional endocannabinoid system that is able to metabolize and bind the two major endocannabinoids AEA and 2-AG [149]. These authors also reported that capacitation remarkably enhances the endogenous tone of both AEA and 2-AG through distinct mechanisms [149]. One interesting interaction has been reported between endocannabinoid and nitric systems, in that AEA would be implicated in the regulation of sperm release from the oviduct and sperm capacitation in bovines by activating the NO pathway in both sperm and the oviductal epithelium [150].

More recently, an unconventional endocannabinoid signaling (1-AG/2-AG), related to progesterone and calcium influx, has been described for the sperm activation [151]. Specifically, the authors showed that α/β hydrolase domain-containing protein 2 (ABHD2) metabolizes endocannabinoids 1-AG/2-AG only in the presence of progesterone, and interestingly these endocannabinoids act as endogenous inhibitors of CatSper [151]. The authors suggest a model in which AGs presence before the progesterone would have the role of keeping blocked the CatSper channel and all the cellular events mediated by their specific influx of calcium, particularly the sperm hyperactivation. The action of AGs is relevant if considering that progesterone induces Ca^{2+} influx into spermatozoa and triggers multiple Ca^{2+}-dependent physiological responses essential for successful fertilization, such as sperm hyperactivation, acrosome reaction, and chemotaxis toward the egg [152], and a very interesting antecedent is that progesterone is still secreted by the cumulus of ovulated eggs [111], and of course the sperm interaction with COCs complexes would be the physiological context for the full sperm activation. On the other hand, electrophysiological studies showed in human spermatozoa that nanomolar concentrations of progesterone dramatically potentiate CatSper [152].

Another important effect indirectly linked to CatSper but mediated by endocannabinoids is the activation of Voltage-Gated Proton Channel (Hv1) by AEA. The Hv1 is a fast regulator of intracellular pH in human sperm [153]. It is known that CatSper channel is strongly potentiated by intracellular alkalization. On the other hand, Hv1 and CatSper channels are located in the same subcellular domain and their combined action can induce elevation of both intracellular pH and Ca^{2+} two signals required for sperm activation in the female reproductive tract [154]. It is very interesting that the two major endocannabinoids (AGs and AEA) can have opposite effects on CatSper channel functionality through nonreceptor-dependent mechanisms [151,153].

The presence of AEA in seminal plasma and uterine fluids would have a preventive effect on the premature capacitation process of fresh sperm. Sperm that have gone through the uterus and oviduct would be exposed to a gradual reduction of AEA, releasing the "molecular brake" of acrosomal secretion by inhibitory action of CB1 receptor [60,144]. In this context, it should be noted that AEA is synthesized in the oviduct and uterus [155,156], and the levels of AEA in uterus are higher than in any tissue examined [155]. In this manner, all of the described results strongly point out the importance of the endocannabinoids in the regulation of sperm function inside the female genital tract.

Melatonin signaling
Melatonin (N-acetyl-5-methoxytryptamine) is an indoleamine, which is synthesized from tryptophan. Melatonin has been identified as a key factor in the regulation of circadian rhythms and the sleep-wake cycle [157]. Long exposure to artificial lighting leads to a reduction in endogenous melatonin exposure [158].

Circulating melatonin in vertebrates is mainly secreted by the pineal gland in response to suprachiasmatic nucleus activity, according to light/dark photoperiod, with a nocturnal maximum and a diurnal basal level, and determines circadian rhythms such as the sleep cycle and body temperature [159,160]. Furthermore, it has been claimed that melatonin has a large number of functions including sugar and lipid metabolism [161,162] and oxidative stress control [163]. Melatonin also acts as a universal reactive oxygen species (ROS) and reactive nitrogen ones scavenger [164–166]. Melatonin also regulates reproductive seasonal variation in many animal species [167,168]. Depending on the species (long-day or short-day breeders), melatonin can act as an antigonadotropic or progonadotropic hormone [169,170].

Several empirical studies have shown that the use of melatonin has a direct beneficial effect on sperm cells. A strong association between sperm quality and high levels of endogenous melatonin has been reported in humans. Thus, in vitro treatment with melatonin can improve human sperm motility [171] and several quality parameters of ram [172] and boar sperm [173]. Known subcellular effects of melatonin in sperm physiology include a decrease of oxidative damage through lowering the intracellular levels of ROS and NO [173–175], as well as a reduction of lipid membrane peroxidation [176,175], intracellular apoptosis markers signals [177], and DNA fragmentation [178].

Likewise, melatonin has been used as an additive in refrigeration and cryopreservation extenders, specifically improving the refrigeration sperm quality in boar [179], red deer [180], and sperm freezability in ram [181], bull [182], and stallion sperm [183]. All these positive effects of melatonin on sperm have been tested in seasonal mammals (horse, ram, and deer) as well as in nonseasonal ones (human and boar), suggesting that this action is unrelated to that exerted by the hypothalamic–pituitary–testicular axis regulation [159].

The action of melatonin on sperm cell has been related to their free radical control properties and its solubility property to cross the plasma membrane, thus reducing the levels of oxidative stress in both the extra- and intracellular media [184]. Moreover, high levels of melatonin have been found in ram seminal plasma during the reproductive season and during the nonreproductive season after melatonin implantation [185]. Melatonin could protect ejaculated sperm from oxidative damage due to its antioxidant properties and its stimulation of the seminal plasma antioxidant defense system [159]. Similarly, studies in other species suggest the protective effects of melatonin on semen. Effectively, Jang et al. showed that supplementation of melatonin in boar sperm extend could improve the sperm quality and increase the blastocyst production index, independent of the exposure to hydrogen peroxide [173].

Melatonin binds to specific receptors on the cell surface. Melatonin receptors type 1 and 2 (MT1and MT2) belong to the seven transmembrane G-protein coupled receptor (GPCR) family [186]. Both receptors are linked to the inhibition of cAMP production [186,187].

The signal transduction pathways activated by MT1 and MT2 are complex. In this sense, the MT1 receptor activation increases the phosphorylation of the cAMP-activated PKA [188,189] and PKC
Serotoninergic system

Serotonin (5-hydroxytryptamine or 5-HT) has been detected in human semen [201]. These authors showed that men with blood serotonin levels greater than 100 ng/ml had lower sperm counts and sperm motility than those with normal levels of blood serotonin (less than 90 ng/ml). When blood serotonin increased from 50 to 90 ng/ml (normal range), there was an increase in the percentage of sperm with excellent motility. Other study confirmed that hyperserotoninaemia was related to both diminished sperm count and sperm motility [202].

Both serotonin and 5-methoxytryptamine, a serotonin receptor agonist, can initiate the acrosome reaction of epididymal hamster sperm. On the contrary, both Quipazine, a 5-HT3 antagonist [203], and cyproheptadine, a 5-HT1 receptor antagonist [204], inhibited the acrosome reaction initiation [205]. More recently, the previous findings were confirmed, where elevated serotonin endogenous levels, indirectly assessed as urinary 5-hydroxy indoleacetic acid, were correlated with poor sperm quality that, consequently, lead to subfertility problems [206]. Two years later, a full characterization of the components of the serotoninergic system was reported in human sperm [71]. In this work, the authors showed the presence of serotoninergic markers such as serotonin, tryptophan hydroxylase 1 (TPH1), monoamine oxidase A (MAO-A), all the known serotonin receptor subtypes (5-HT1B, 5-HT2A, and 5-HT3), serotonin transporter (5-HTT or SERT), and TPH enzymatic activity. Furthermore, serotonin at a concentration range of 10–100 μM increases motility and induces tyrosine phosphorylation in human sperm [71]. In addition, we have shown the presence of SERT and MAO in bovine and equine sperm [207]. Finally at the functional level, the dual effect of serotonin on sperm motility could be explained by the specific action of serotonin receptor or transporter, at low and high concentration, respectively, as was described for dopamine in both boar and equine sperm [59,74].

Noradrenergic/adrenergic signaling

Several reports on the effects of epinephrine and norepinephrine on sperm physiology suggested the presence of adrenergic receptors in mammalian sperm. Both adrenal gland extracts and purified catecholamines were reported to stimulate hamster sperm motility (either general or hyperactivated), capacitation, acrosome reaction, and fertilizing ability in vitro [208–213].

Some possible functions assigned to norepinephrine could be the transport of sperm through the vas deferens [214] and contraction of the seminal vesicles [215]. Sperm function studies have indicated that norepinephrine stimulates sperm capacitation in hamster [211,212] and bovine sperm [216], as well as stimulating in vitro motility in hamster sperm [210] and promoting the acrosome reaction in hamster [209,212] and bovine sperm [216]. On the other hand, the inhibitory effect of epinephrine has been reported on sperm capacitation and acrosome reaction [209,213]. In the same line of evidence, the effects of β-adrenergic drugs in enhancing sperm motility have also been reported [210,217].

The presence of different adrenergic receptors in sperm, including α-2 and β-adrenergic receptors in mouse and human sperm [72,73] has been shown. These authors consistently showed that norepinephrine induces capacitation in mouse sperm [72].

Another highlight is the biphasic effect of norepinephrine on capacitation and acrosome reaction parameters that are favored or inhibited at low (3.1 μM) and high (3.1 mM) doses, respectively [216]. At physiological level, high levels of norepinephrine have been detected in human and cow oviduct compartments [218,219]. Specifically, high concentration was found in the isthmus, region that contains adrenergic nerves related to smooth muscle [218], suggesting a role in sperm capacitating or fertilization control. Moreover, high levels of norepinephrine have been detected in bovine oviductal and follicular fluid [216].

Finally, all evidence reinforces the idea that norepinephrine can mediate functions in sperm by independent way of α- or β-adrenergic receptors, involving the norepinephrine transporter (NET). NET and other catecholamine transporters, such as dopamine transporter (DAT), have been immunodetected by western blot and immunocytochemistry in stallion, bovine, and human sperm [59].

Dopaminergic system

In mammals, the female reproductive tract is innervated by autonomic nervous system, predominantly adrenergic fibers [220–222]. In contrast to other organs with adrenergic innervation, the uterus is affected by steroid hormones during the estrous cycle and pregnancy [223,224]. This innervation is important in the neuronal control of uterine blood pressure, myometrial contraction, and secretory endometrial function [225] and exerts biological effects consistent with a regulatory role in female reproduction [226].

Both tyrosine hydroxylase (TH) as dopamine-β-hydroxylase (DbH) are present in uterine innervations [221,227], and are present in the myometrium and endometrium belonging to all regions of the uterus and cervix [228]. These fibers (TH positive and DbH) are not only restricted to the uterus but also are found in the oviduct [229] and the ovary [230]. This evidence suggests a regulatory role for catecholamines in organs and female reproductive tissues, not only related to the oviductal and uterine contraction but also in functions associated with transit of gametes and embryos in the different.
regions of the oviduct. High levels of dopamine were described in the human ovary [231]. It has been reported that monkey oocytes are able to take up dopamine and use it as a precursor for the synthesis of norepinephrine by DβH [232]. Moreover, noradrenaline, TH, and DβH have been detected in different parts of the rabbit oviduct [233, 234].

Dopamine type 2 receptor (DRD2) has been detected in testis, spermatogenic cells, and sperm in rats as well as in human, bull, and mouse [38]. Later, Ramírez et al. showed that boar sperm express DRD2, and that at functional level, their activation with dopamine (100 nM) has a positive effect on cell viability, parameter that was correlated with AKT/PKB signaling pathway [74]. On the other hand, these authors reported that bromocriptine (100 nM) and dopamine (100 nM and 10 μM) increased tyrosine phosphorylation and sperm motility during in vitro capacitation, and showed a biphasic effect [74]. This biphasic effect was similar to that already described for epinephrine in bull spermatozoa [216] and with serotonin in humans [202]. A dose-dependent effect on boar sperm function was proposed. First, at low level (<10 μM) dopamine increases viability, tyrosine phosphorylation, and motility by DRD2 activation. At high levels, dopamine (1 mM) decreases tyrosine phosphorylation and motility by a mechanism involving dopamine uptake by a DAT by cytotoxic accumulation of dopamine or by enzymatic oxidation or autooxidation of dopamine [74]. Biochemical studies support that hypothesis in which western blot and immunocytochemical analyses in human, mouse, and equine sperm suggest the presence of the DAT [74].

More recently, the presence and functionality of DAT in equine sperm has been confirmed [59]. These authors showed that equine sperm have a functional dopamine transporter using 4-[4-(Dimethylamino) styryl]-N-methylpyridinium iodide (ASP+), as a dopamine analog substrate in uptake assay. Moreover, they also showed in boar sperm [74] that dopamine (1 mM) treatment in vitro does not affect sperm viability but decreases total and progressive sperm motility. This effect was reversed by DAT blocking with vanoxerine (GBR12909), the selective DAT inhibitor and nomifenine and bupropion, a nonselective DAT inhibitor [59]. The authors demonstrated that high concentrations (1 mM) of dopamine decrease both acrosome integrity and tyrosine phosphorylation, confirming the biphasic effect of dopamine in phosphotyrosine, acrosome integrity, and sperm motility [59].

The presence and functionality of DR2R and DAT described above suggest a high degree of conservation of the mammalian dopaminergic system and the participation of dopamine in the control of the overall mammalian sperm function. Other studies performed in different portions of human fallopian tubes (isthmus, ampulla, and fimbriae) showed that the highest concentration of catecholamines is found during the preovulatory and ovulatory phases [218]. Finally, it is possible to hypothesize that variations in catecholamine concentrations could take place in microdomains from mammalian oviduct (ampulla and isthmus region), thereby regulating the sperm capacitation state before, during, and after the ovulation event.

Synthesis and conclusions

Classically, sperm function in in vitro analysis associated with neural signals has been analyzed as an unidimensional approach (single ligand-receptor effect). However, the sperm cells in the ejaculate and in the female reproductive tract are exposed to multiple neural signals in a multidimensional signaling context. In this regard, sperm, unlike neurons, would be highly differentiated cells from the structural and functional point of view but with a low level of signaling specialization, because they present at the same time a repertoire of GABAAergic, dopaminergic, noradrenergic, purinergic, cholinergic, serotonergic, melatoninergic, receptors to name a few. On the other hand, and from an evolutive point of view, the sperm cell does not have a functional genomic feedback, representing a complex cellular model that maximizes the use of signaling transduction platforms that are activated by several “input signals,” as ligands–receptors classic interactions, receptors transactivation, and homodimerization events.

G protein signaling

The cAMP plays a central role in cell signaling in a wide variety of cell types. This cyclic nucleotide is synthesized by several adenyl cyclases (ACs) that can generally be grouped into two types: transmembrane ACs and soluble ACs. With regard to sperm, several aspects of their physiology are regulated by cAMP, which is synthesized through a single atypical AC (Adcy10, aka sAC, SACY). The signature that identifies sAC among other ACs is its direct stimulation by bicarbonate. The essential nature of cAMP in sperm function has been demonstrated using gain of function and loss of function approaches. In this sense, it is known that cAMP and the enzymes involved in cAMP signaling pathways are required for the acquisition of fertilizing capacity of mammalian sperm [235].

Receptor-mediated inhibition of adenylate cyclase requires the presence of a guanine nucleotide-binding protein complex, named Gαs. The inhibitory G protein (Gi) can be functionally inactivated as a result of an ADP-riboseylation modification catalyzed by pertussis toxin [236]. Sperm from invertebrates and vertebrates contain a membrane-bound protein that is ADP ribosylated by pertussis toxin, but not by cholera toxin [237], situation that reveals the absence of the stimulatory G protein type (Gαs). However, recent evidence has shown the presence of Gαs in mouse spermatozoa. Specifically, Wertheimer et al. reported not only ADP ribosylation by cholera toxin but also Gαs immunoreactivity by western blot and immunocytochemistry. These authors reported that Gαs protein is localized in the acrosome region [238]. Concomitantly, Gαs-proteins are confined to a functionally defined region in the head and tail of spermatozoa [237]. The existence of several G protein types in mammalian gametes suggests their possible involvement in the regulation of various effectors systems, in a manner reminiscent of somatic cells [237].

In human spermatozoa, the presence of Gαo2, Gαi2, Gai/q213, and Gαs22 proteins has been reported by immunological methods. In regards to localization pattern, the evidence indicate that Gαi2 is present in the acrosome region, the midpiece, and tailpiece, and Gαs1 is present in the postnuclear cap, midpiece, and tailpiece regions [239]. On the other hand, Gαq has been localized in the acrosome, midpiece, and tailpiece, Gαs3 subunit was localized in midpiece and the equatorial segment of the acrosome [237, 239]. The distribution pattern of G proteins suggests that they may couple to receptor or effectors, which also show discrete regions of localizations in spermatozoa [239, 240].

With regard to G protein signaling, almost all mentioned receptors have a functional coupling to the inhibitory G protein type (Gαi1/2). This situation suggests a type of negative regulation on cAMP accumulation, as in the case of GABAB receptor [241], μ- and δ-opioid receptor [242], CB1 cannabinoid receptor [236], DRD2 dopamine receptor [243], MT1 and MT2 melatonin receptors [244, 245], HT1B serotonin receptor [246], and α-2 adrenergic
receptor [247]. Moreover, there is the possibility that some receptors increase the molecular complexity of GPCR signaling by heterodimerization events, in three ways:

(i) Binding properties: Many studies have reported that receptor heterodimerization leads to new binding properties, as in the case of μ- and δ-opioid receptor heteromers for several agonists [248], suggesting that heteromerization induces an alteration in the conformation of the ligand-binding site [249].

(ii) G protein activation: The μ- and δ-opioid receptor association decreases μ-opioid receptor activity in response to selective agonists [250]; the μ-opioid receptor association with α-2 adrenergic receptor increases its activity [251], and their association with CB1 does not affect μ-opioid receptors activity [252].

(iii) It has been shown that μ- and δ-opioid receptor heteromers are ligand independent (i.e., the receptors in the heteromer are coupled to distinct G proteins without ligand stimulation). Although both μ- and δ-opioid receptors couple to Gαo, when individually expressed, the heteromer has been shown in some cases to selectively couple to Gαs [253]. In addition, heteromerization between CB1 and DR2R involves a switch in CB1 coupling from Gαo to Gαs upon co-activation of DR2R [254]. Furthermore, the activation of either DR2R or CB1 in HEK-293T cells co-expressing both receptors leads to the inhibition of forskolin-stimulated cAMP accumulation, whereas the co-activation of both receptors results in a loss of cAMP inhibition [255]. The strong association described between DR2R and CB1 receptors was confirmed in a nonheterologous system, specifically shown in Figure 1. Double immunohistochemistry and co-immunoprecipitation analyses performed in rat nervous tissue suggest an interaction between DR2D and CB1 receptors. The immunoprecipitation analysis was also positive when using protein extracts from rat testis and rat and human spermatozoa (Figure 1).

Figure 1. (A–E) Double Immunohistochemistry of DRD2 (green) and CB1 (red) receptor in striatal region (rat brain) by confocal microscopy. Low magnification and merged color images are shown in panels A and B; negative control is shown in inset in panel A; high magnification (from inset in panel B) and individual channel and merged images are shown in panels C, D, and E. Both arrowhead and arrows show a strong colocalization pattern of both receptors in body and neural projection, respectively. Scale bar, 10 μm. (F) Co-immunoprecipitation analysis of DRD2 and CB1 receptors using protein extracts from brain, testis, and spermatozoa. Protein lysates were co-immunoprecipitated (IP) with anti-CB1 receptor (rabbit polyclonal, C8985 Sigma Chemical, Co) and protein A-Sepharose and immunoblotted for DRD2 receptor (sc-9113, Santa Cruz Biotechnology). Each line corresponds to sample obtained from control without protein (1) at brain (2), rat testis (3), rat spermatozoa (4), and human spermatozoa (5). Taken with permission from Ramirez [329].

Because receptor oligomerization often affects binding, activity, and signaling of GPCRs, the formation of receptor heteromers has been used as an explanation for many of the described crosstalks involving these receptors. However, it has been recently reported that crosstalk could result not only from receptor oligomerization, but also from colocalized receptor sharing signaling pathways, or from synergistic regulation of signaling crossroads, independent of oligomerization [259]. Interestingly, if the interaction between two GPCRs was not already complicated to decipher recent evidence suggests the possibility of a triple crosstalk between three GPCRs present in sperm cells: DRD2 [58], metabotropic glutamate receptor (mGlu5) [260], and adenosine receptors (A2A) [261]. This crosstalk would act in the regulation of the striatal pathway as a very fine and sophisticated modulating system [262]. On the other hand, spermatozoa contain both soluble and membrane-bound forms of adenylyl cyclase [263]. Since the soluble isofrom can be activated by bicarbonate [264], the presence of Gαs protein in spermatozoa [238], but absence of GPCRs coupled to Gαs, suggests that cAMP produced in the sperm cell comes exclusively from a non-G-protein-dependent pathway. Future research will be necessary to elucidate the role of GPCRs in the production and control of cAMP in the physiology of sperm capacitation.

Specific signaling that involves Gαq/11 protein seems to be more limited than Gαs, The Gαq/11 protein coupling would be a chemical restraint to serotonin receptor activation of 5-HT2A and 5-HT1B. This restrain could explain the effects of serotonin on the motility and tyrosine phosphorylation profiles in human spermatozoa [71]. Future studies are needed to better understand the specific coupling that triggers the effects of serotonin in sperm physiology: Gαq/11, or both signaling pathways.

The Gβγ subunit has been less studied than Gα. The β-γ complex can be formed by 5 different β subunits and 12 γ subunits [263], Gβγ signaling has been linked with ion channel regulation, such as G protein-gated inward rectifier channels, as well as calcium channels [266]. Another example of Gβγ signaling is its effect of activating or inhibiting AC leading to the intracellular increase or decrease of cAMP [267]. Interestingly, it has been reported that G protein β-γ subunits activated by serotonin are involved in presynaptic inhibition by regulating vesicle fusion properties [268], through a mechanism that involves direct interaction between G proteins and the vesicle fusion machinery, specifically with SNAP-25 in SNARE complexes [269,270]. Regarding sperm physiology, these important findings
may explain the inhibition of the acrosome reaction, process that is molecularly controlled by SNARE complex [271], by the activation of several GPCRs: GABA$_\text{A}$R [101], CB1 [82,83] and α-2 adrenergic [73]. On the other hand, the G$_{\text{Gq/11}}$ dimer was found to play a putative role in the activation of effectors such as PLC$_\beta$, PI3K, and Src. All these associations between trimeric G proteins and second messengers lead to a cascade of intracellular events that cause a particular response, depending on cell type.

The phosphoinositide 3-kinase (PI3K) pathway can be activated by receptor tyrosine kinases (RTKs), or by integrin transactivation or GPCRs [272]. It is unclear how G proteins activate PI3K, but it is known that PI3P$_2$ is converted to PI3P (capable of activating Akt) by PI3K, whereas PTEN opposes this reaction by dephosphorylating PI3P. The role of G$_{\text{Gq/11}}$ subunit in PI3K activation in somatic cells has also been reported, and it is known that there is a direct activation of kinase by the βγ dimer [272]. In sperm cell, the PI3K/AKT pathway activation by neurotransmitter has not had enough attention. Western blot analysis of boar sperm capacitation in the presence of 100 nM dopamine suggests that this pathway could be involved in sperm viability. The pattern of phospho-AKT localization suggests a possible function in the acrosome reaction, energetic control, and motility [74]. It is possible that many of the effects assigned to GPCRs activated by neurotransmitter receptors in sperm functionality involve the βγ dimer, in particular those related to cell survival (via PI3K/Akt), the acrosome secretion-mediated SNARE machine, and sperm motility and their relationship with energy metabolism.

Transactivation of G-protein coupled receptor-receptor tyrosine kinase

The activity of most GPCRs is regulated by GPCR kinases that phosphorylate C-terminal tail of activated GPCRs, preventing further interaction with heterotrimeric G protein, and leading to termination of receptor signaling and receptor desensitization [273]. Additionally, RTKs and GPCRs act in concert to regulate physiological processes, and in some cases their effects are synergic, whereas in others they antagonize [274]. Activation of GPCRs can stimulate the signaling activity of RTKs connecting the broad diversity of GPCRs with the potent signaling capacities of RTKs. This molecular mechanism is termed “transactivation,” and was first described in Rat-1 fibroblasts stimulated with a number of GPCR agonists, which induced a rapid tyrosine phosphorylation of epidermal growth factor receptor (EGFR) [275]. The GPCR-induced EGFR tyrosine phosphorylation is rapid, transient, and comparable with receptor activation by low amounts of EGF [276].

Transactivation of the EGFR by GPCRs is usually accomplished through a so-called triple-membrane-passing-signal pathway [277,278]. During this cascade, stimulation of a specific GPCR, e.g., a mAChR, is followed by the activation of a metalloproteinase that proteolytically cleaves a membrane-bound precursor of a ligand of the EGFR family. Extracellular release of an active ligand capable of activating the EGFR will induce a downstream signaling response mediated by EGFR and the MAPK cascade. Depending on the cell type, the molecular details of this transactivation process show considerable flexibility [279]. In some cells, additional intracellular signaling cascades involving, e.g., the Src kinases, phosphatidylinositol 3-kinase, or PKC, are activated in parallel to the MAPK pathway, resulting in modulation of the downstream response [280].

Activation of the EGFR generates a Ca$^{2+}$ signal, broadly defined as the transient rise of the intracellular concentration of Ca$^{2+}$ [281]. EGF has been detected in human semen [282], and their receptor EGFR was detected in bovine spermatozoa, localized in the head and midpiece [283,284]. It has also been shown that EGFR is involved in the AR and in actin polymerization during sperm capacitation [283–285]. Moreover, EGFR phosphorylation/activation is increased during capacitation. Further stimulation of the EGFR in capacitated sperm reveals increased intracellular calcium levels leading to AR [283].

To date, the sperm EGFR transactivation mediated by activation of GPCRs has been suggested for α-7 acetylcholine receptor (AChR) [286], angiotensin II receptor type 1 (AGTR1) [283], and DRD2 [74]. However, the EGFR transactivation by GPCR activation has been reported in somatic cells with the use of serotonin, norepinephrine, and neurotensin, through 5-HT1A [287], α2AR [288], and neurotensin receptor [289], respectively.

Other RTKs in somatic cells have shown transactivation mediated by GPCRs agonists: the NGF receptors TrkA by adenosine [290], TrkB and PDGFR by serotonin [291], and FGFR receptor by endocannabinoid [292] and μ-opioid agonist [293]. Interestingly, FGFR and PDGFR have been reported in spermatozoa suggesting its involvement in motility regulation [294].

**Biphasic effect or intracellular effect mediated by transporters of ligands**

Evaluation of sperm function parameters in response to different concentrations of agonists has demonstrated that many of the neurotransmitters studied generate a “biphasic” response, which turns out to be paradoxical, or at least contradictory at first. Several neurotransmitters generate a response at low doses by GPCR activation, and quite another response that involves the intracellular incorporation of the agonist by a specific transporter activity. This biphasic response has been reported by lipophilic neurotransmitters as cannabinoids agonist and hydrophilic neurotransmitter agonists [64,71,81,142,144,202]. In this context, the hydrophilic agonist includes the monoamines: melatonin and serotonin (indoleamines) and dopamine and norepinephrine (catecholamines), able to alter several parameters in a dose-dependent manner and by GPCR receptor activation at low doses and intracellularly after incorporation via specific transporters.

With regard to sperm function, a biphasic effect of both dopamine and serotonin on sperm motility, which was increased directly by submillimolar doses, and tyrosine phosphorylation has been reported [71,74,202]. Both effects could be mediated by the action of GPCR receptor DRD2 (short or/and long isoform) and 5-HT (1B or/and 2A), respectively. On the other hand, the inhibitory effect observed on the same parameters at supramillimolar doses of dopamine in boar and stallion spermatozoa [59,74], could be mediated by intracellular accumulation after its incorporation by DAT reported by the same authors [59,74].

The molecular mechanism by which intracellular accumulation of dopamine causes inhibition of sperm motility is unknown. However, it is known that dopamine is a very reactive molecule compared with other neurotransmitters, and dopamine degradation naturally produces oxidative species [295]. More than 90% of dopamine in dopamine neurons is stored in abundant terminal vesicles and is protected from degradation [296]. However, a small fraction of dopamine is cytosolic, and it is a major source of dopamine metabolism and presumed toxicity. Cytosolic dopamine undergoes degradation to form 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid, as well as hydrogen peroxide via the monoamine oxidase pathway [295,297]. Alternatively, dopamine undergoes
Immunodetection and enzymatic activity of MAO-A in equine spermatozoa. Western blot analysis (A), immunofluorescence analysis (B). MAO activity was determined fluorometrically for 4-hydroxyquinoline production and using a kynuramine dihydrobromide as substrate, *P < 0.01, one-way ANOVA. (C) Heterogenic “in situ” detection of MAO activity by use of tryptamine and tetrazolium blue: negative control (D), positive spermatozoa for the test (F). Bars scale: 10 μm. Taken with permission from Urra [207].

oxidation to form superoxide, hydrogen peroxide, and o-quinone or reacts with cysteine residues on glutathione or proteins to form cysteinyl-dopamine and cysteinyl-DOPAC conjugates [298–300].

The biphasic effect associated with norepinephrine has been reported for sperm capacitation and acrosome reaction parameters in bovine [216]. The α-2 adrenergic receptor reported in mouse and human spermatozoa could play a central role [72,73] as well as the dopamine transporter NET, recently reported by Urra et al. [59].

Dopamine generates 3,4-dihydroxyphenylacetaldehyde (DOPAL), whereas both norepinephrine and epinephrine are deaminated to form 3,4-dihydroxyphenylglycolaldehyde (DOPEGAL) [301]. Both DOPAL and DOPEGAL are generated by MAO-A activity. In fact, there is evidence demonstrating the neurotoxic properties of the catecholamine-derived aldehydes DOPAL and DOPEGAL by various cytotoxic mechanisms including the generation of free radicals or ROS and the initiation of apoptosis [302,303]. It is possible that supramillimolar doses of norepinephrine, DOPEGAL, or other oxidized derivatives may be sublethal on sperm physiology, impacting only motility, sperm capacitation, and acrosome reaction, but not sperm viability.

Physiologically, there are reports revealing that the seminal plasma contains norepinephrine, epinephrine, L-DOPA, and DOPA [304]. Hence it is interesting to note that the concentrations described, at least L-DOPA and noradrenaline are 6 and 42 times higher, respectively, than those determined in blood plasma [304], suggesting that norepinephrine in vivo could have a control role on acrosome reaction, preventing their premature activation.

In the case of serotonin, the hyperserotoninemia was related to diminished sperm motility [303]. A similar biphasic effect has been reported for hyperactivity sperm motility in hamster by serotonin agonists [305]. Conversely, the presence of serotonin transporter (5-HTT or SERT) in spermatozoa [59,71] suggests that these effects are mediated by intracellular action of serotonin. It is also possible that sperm incorporate tryptophan, and that serotonin is metabolized by the enzyme TPH1, as reported in human spermatozoa [71]. Serotonin, like other biogenic monoamines, is degraded by the two known isoforms of MAO, MAO A and MAO B, which show differences in their tissue and cellular distribution [306]. In this context, the presence and activity of MAO have been reported in equine spermatozoa [207] (Figure 2). Furthermore, the presence of 5-HTT (SERT) and MAO has been also reported in human spermatozoa [55]. It is suggested that excessive activity of these enzymes may lead to mitochondrial damage in neural cells, as well as in other cell types [307,308]. Moreover, hydrogen peroxide produced by oxidation of monoamine substrates can induce oxidative stress resulting in neuronal death [309]. Serotonin is oxidized by superoxide [310] or myeloperoxidase [311], forming a reactive quinone, tryptamine-4,5-dione (TD), and a dimer of serotonin. The TD covalently reacts with the thiol [312,313], and inactivation of enzymes via the formation of quinone adducts has been reported [310,314]. Kato et al. have reported in an in vitro study that cytoskeletal proteins are targets of TD, using an immunological approach [315]. An overview of actors and effects on sperm functionality described for monoamines (dopamine, norepinephrine, and serotonin) is shown in Figure 3.

Melatonin formation from 5-HT requires two pineal/retina-specific enzymes: serotonin N-acetyltransferase, which forms N-acetylserotonin (NAS) from serotonin, and hydroxyindole-O-methyltransferase, which produces melatonin from NAS [316]. The presence of these enzymes has not been reported in testis or spermatozoa; however, the presence of such metabolic repertoire would assign a pivotal role for serotonin in the ROS production and quinone-proteins balance, reducing the levels of oxidative stress by their conversion to melatonin.

Melatonin is a derivative of the amino acid tryptophan and easily crosses biological membranes due to its amphipathic nature.
However, the mechanism by which melatonin enters into cells remains unknown. Changes in redox state, endocytosis pathways, multidrug resistance, glycoproteins, or a variety of strategies have no effect on melatonin uptake [317]. Classically, it has been considered that melatonin enters freely into most of cells by passive diffusion through the cell membrane. However, there are data that support the hypothesis that a facilitated diffusion or an active process rather than simple passive diffusion through the cell membrane is the major mechanism in melatonin uptake [318]. However, to date there are no reports of the existence of specific melatonin transporters. It has been recently demonstrated that members of the SLC2/GLUT family glucose transporters have a central role in melatonin uptake [317], specifically, glucose transporter 1 (GLUT-1). In this sense, the effect of low doses of melatonin on ram sperm capacitation can be mediated by the action of MT1 or MT2 receptors [86], and their signal transduction or their specific properties to reducing the levels of oxidative stress in both the extracellular and intracellular media [184]. Besides, the ubiquitous presence of GLUT-1 in mammalian spermatozoa [319] would explain the uptake and intracellular effects of melatonin in sperm control of intracellular ROS. Several studies have shown a protective effect of melatonin regarding control of damage induced by ROS both extracellularly and intracellularly [159,173–175,184,200,320]. In that sense it is not unreasonable to think that melatonin in vivo could act as a buffering agent of redox state, which varies according to the stage of sperm functionality and molecules present in seminal plasma, uterus, and oviduct, particularly those that participate in ROS generation as monoamines: dopamine, norepinephrine, epinephrine, and serotonin. However, future studies will be needed to clear both the spatial and metabolic interaction of melatonin and monoamines in the sperm functionality.

Oxidative stress has been considered a major contributing factor to mechanisms that limit the functional competence of mammalian spermatozoa involving lipid peroxidation, induction of oxidative DNA damage, formation of protein adducts, and consequently infertility. However, it is clear that ROS are important contributors to the regulation of sperm function in both a positive and a negative sense [321]. Incubation of sperm cells with low concentrations of hydrogen peroxide has been found to stimulate sperm capacitation, hyperactivation, acrosome reaction, and oocyte fusion [322–324] and high concentrations of the ROS cause sperm pathology (ATP depletion) leading to insufficient axonemal phosphorylation, lipid peroxidation, and loss of motility and viability [325].

Finally, it is clear that normal sperm function depends on low levels of ROS generation in order to promote the signal transduction pathways associated with capacitation [321]. Future research efforts should be directed toward understanding the role of neurotransmitters (dopamine, norepinephrine, serotonin, and melatonin) and their subproducts and derivatives or effectors (ROS, aldehydes, quinones, and others) on the regulation of sperm function.
or quinoproteins) in the redox balance and synchronicity during the different phases experienced by the sperm functionality.

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