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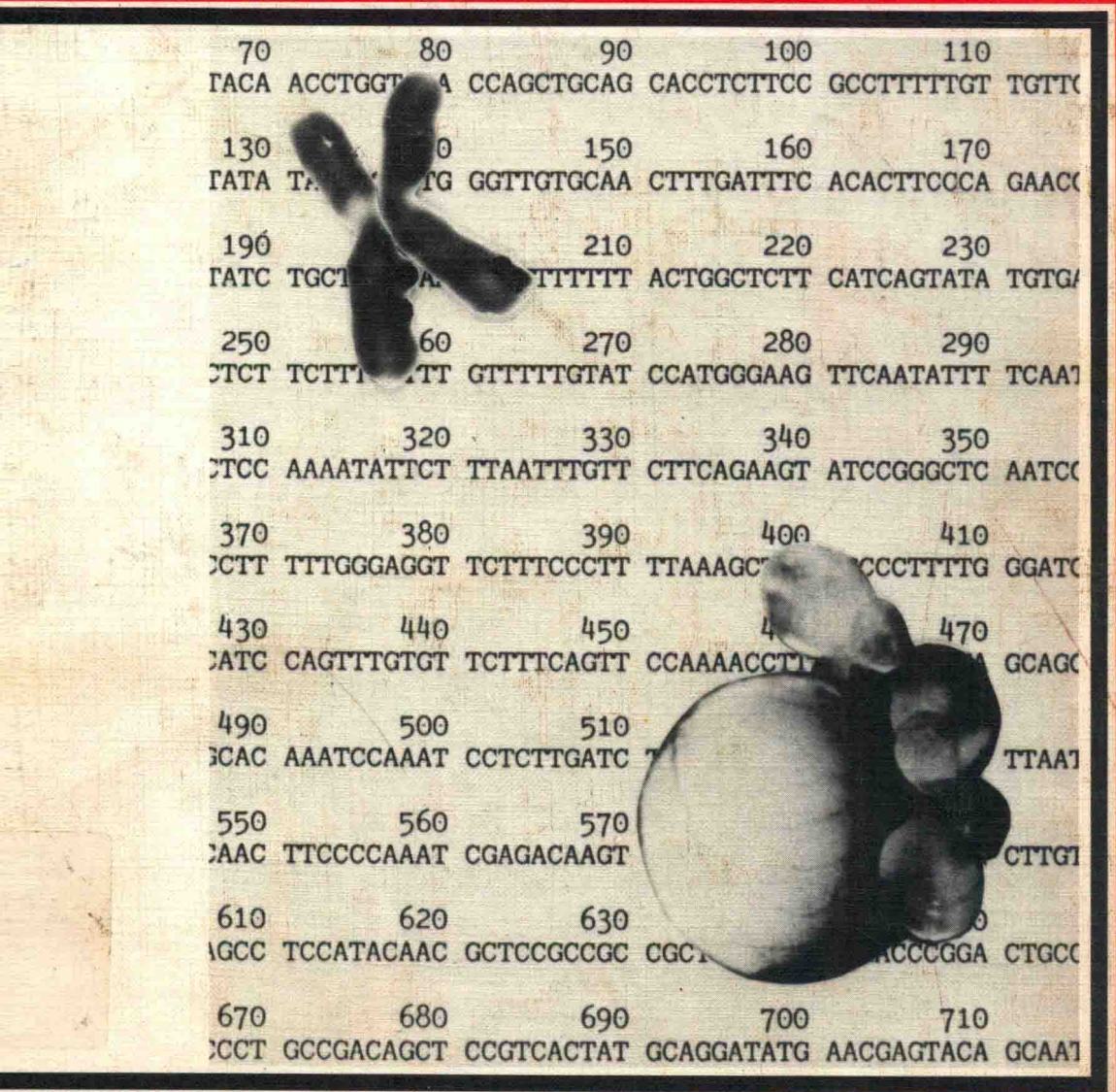
Cholinesterase Genes: Multileveled Regulation



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Cholinesterase Genes: Multileveled Regulation

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I. Introduction: Multileveled Regulation of the Human Cholinesterase Genes and Their Protein Products

1. Overview and General Significance

Acetylcholinesterase (AChE) is an enzyme long noted for its essential role in the termination of neurotransmission at cholinergic synapses and neuromuscular junctions. As the target protein for a variety of neurotoxic compounds, including common agricultural insecticides and chemical warfare agents, research on this enzyme has profound implications for human health and well-being. A member of the highly polymorphic family of cholinesterases (ChE), proteins expressed in tissue-specific and differentially regulated fashions, AChE presents a powerful model for the basic scientific study of complex gene regulation and divergent pathways in protein biosynthesis.

Recent accumulated evidence, covered in a number of excellent reviews, has revealed the primary structure of human ChEs and the chromosomal localization of the CHE1 gene encoding the related enzyme butyrylcholinesterase (BuChE). Further analytical expression studies have demonstrated that the biochemical properties of human ChEs are mostly inherent to their primary amino acid sequence, and the discovery of ChE gene amplifications implicated AChE and BuChE in cholinergic influences on cell growth and proliferation. Abnormal expression of both ChEs, the amplification of the ACHE and CHE genes encoding these enzymes, and unusual BuChEmRNA transcripts have been variously associated with abnormal megakaryocytopoiesis, different leukemias, and malignant tumors of the brain and ovary. The suggestion has been put forth that sublethal exposure to organophosphorous (OP) poisons may, through its effect(s) on ChEs, create a selection pressure for ChE gene amplifications and, in turn, play a role in cancer etiology. This monograph presents the development and outcome of these basic research and clinically oriented studies, within the context of current research advances in the field of ChEs.

The family of ChEs has been the subject of intensive research for five decades, with a continuous increase in the number of studies being focused on this family of enzymes as well as on their scope and diversity [63, 333]. At the biochemical level, ChEs are highly polymorphic enzymes of broad

substrate specificity [46]. At the biological level, it has been assumed for a long time that ChEs are involved in the termination of neurotransmission in cholinergic synapses and neuromuscular junctions.

According to the accepted classification of enzymes, ChEs belong to the B-type carboxylesterases on the basis of their sensitivity to inhibition by OP poisons [348]. ChEs are primarily classified according to their substrate specificity and by definition of their sensitivity to selective inhibitors into AChE (acetylcholine acetylhydrolase, EC 3.1.1.7) and BuChE (acylcholine acylhydrolase, EC 3.1.1.8) [225]. However, the complete scheme is much more complex. Further classifications of ChEs are based on their electrical charge, their level of hydrophobicity, the extent and mode of their interaction with membrane or extracellular structures and, last but not least, the multisubunit association of catalytic and noncatalytic 'tail' subunits composing together the biologically active ChE molecules [304, 305, 315].

Apart from the neuromuscular junction, ChEs are relatively concentrated in the brain. Therefore, ChEs in the mammalian brain have been studied extensively for many years. Colocalization of AChE with choline acetyltransferase in cerebral regions has indicated cholinergic and cholinoceptive properties to certain families of mammalian brain neurons [202]. Also, correspondence was shown between the dopamine islands and AChE patches in the developing striatum [139]. An immunohistochemical study of neuropeptides in cat striatum demonstrated that neurons, producing opiate peptides, are arranged to form mosaic patterns in register with the striosomal compartments visible by AChE staining [140]. Also, AChE immunoreactivity coexists with that of somatostatin in rat cerebral neuron cultures [90]. ChE staining in the mediodorsal nucleus of the thalamus and its connections in the developing primate brain were found to be transient [186], whereas the AChE activity persists to adulthood in the substantia nigra and caudate nucleus of the cat [143] and is subject to in vivo release under electrical stimulation [142] in parallel with dopamine release [71].

What is the role of ChEs in these different brain regions and which elements regulate the expression of CHE genes in the mammalian brain? These questions still remain unanswered. Various studies related AChE activities with neonatal learning [173] and other high functions. Other studies demonstrated hormonal regulation of AChE, for example by ecdysterone [67] in the mammalian brain or by β-ecdysone in *Drosophila* cells [66]. These studies, like the structural related ones, await more detailed molecular analysis.

Numerous studies over the years have indicated that the severe clinical symptoms resulting from intoxication by OP agents [52, 157, 171, 210, 43, 341] are caused by their very tight, irreversible inhibitory interaction with ChEs [5, 181]. OPs are substrate analogues to ChEs, which display

impressive dissociation constants with their catalytic subunit. The labeled OP diisopropyl fluorophosphate (DFP) was shown to bind covalently to the serine residue at the active esteratic B-site region of ChEs, that is common to all of the carboxylesterases [208, 318]. This property has been used in research aimed at protein-sequencing studies, as well as for testing the developmental effects of ChE inhibition [38, 182] or for behavioral studies of OP poisoning [44]. It should be noted that the binding and inactivation capacity of OPs on ChEs is considerably higher than their effect on other serine hydrolases. Furthermore, even within species the inhibition of specific ChEs by different OPs tends to be highly selective to particular ChE types [21, 169].

In addition to their blocking effect on neuromuscular junctions, OPs also act on the central nervous system, sometimes with devastating effects. It is not yet clear why OPs induce seizure-related brain damage [250] although theoretical models were proposed [268]. In order to improve the designing of therapeutic and/or prophylactic drugs to the short- and long-term effects of OP intoxication, it is hence desirable to reveal and compare the primary amino acid sequence and three-dimensional structure of all of the members belonging to this enzyme family, as well as to the homologous domains in other serine hydrolases. Elucidation of these sequences and their interactions within the ChE molecule and with other elements can deepen the understanding of the mode of functioning of ChEs and the specific amino acid residues involved in this functioning. This has therefore been one of the main directions of research in several groups over the past decade. One of the outcomes of this study has been the analysis of sequence similarities between human AChE and related proteins, based on molecular cloning, DNA sequencing and computer analyses of the derived sequences. This analysis revealed, quite unexpectedly, that the genomic sequences encoding AChE and BuChE in humans do not display a high sequence homology, in spite of the considerable similarity between the protein sequences encoded by these two genes.

2. Molecular Form Heterogeneity in Cholinesterases

ChEs constitute a family of carboxylesterase type B enzymes, all of which catalyze the hydrolysis of choline ester compounds at high rates. The localization of such enzymes at cholinergic synapses, where acetylcholine (ACh) is released and must be rapidly inactivated, can be expected. The AChE enzyme present and concentrated at the neuromuscular junction [79, 80] exhibits a high affinity towards ACh. In addition to AChE it has been demonstrated that a high activity of butyrylcholine hydrolyzing

enzyme was present both in the serum [8] and in cholinergic synapses [101]. This enzyme, named BuChE, is also expressed in many additional cell types, including multiple types of embryonic and tumor cells as well as hepatocytes, muscle fibers, endothelial cells and lymphocytes [270]. The role of this enzyme in all of these sites is completely unknown. In addition to ACh, it is capable of hydrolyzing chemically related substrates such as succicurarium and various local anesthetics based on choline esters and related compounds [86, 348]. Because of its high concentration in the plasma and its broad substrate specificity, BuChE is a potentially appropriate scavenger for ecologically occurring poisons that, unless removed at the level of plasma, might get to the brain and induce considerable damage.

In the seventies, different groups [279, 302] studied in detail the molecular polymorphism of both AChE and BuChE in various species, using a variety of biochemical techniques. Several conclusions emerged from this series of studies:

(a) The various globular molecular forms of AChEs and BuChEs include the monomeric, dimeric and tetrameric oligomers of catalytic subunits and are principally composed of catalytic subunits alone [49]. Globular AChE tetramers are subject to selective loss in Alzheimer's disease [18], but it is not yet known what the mechanisms leading to such loss are.

(b) The group defined as 'asymmetric' molecular forms of AChEs includes one triple helical collagen-like catalytic 'tail' subunit associated with one, two or three catalytic tetramers. The collagen-like 'tail' consists of long (50 nm) fibrillary peptides, rich in proline and hydroxyproline residues [280], and is generally assumed to serve as an attachment of its associated tetramers to solid matrix, for example that of the extracellular basal lamina [50].

Several research groups were further able to demonstrate the physical buildup of ChEs at the electron microscopic level, supporting the hypothesis of attachment through the noncatalytic 'tail' subunits. The above model corresponds primarily to the molecular forms of AChEs observed in the electric fishes, namely *Torpedo marmorata* and the electric eel *Electrophorus electricus* [11]. The electric organ of these fishes exhibits very high concentrations of both the nicotinic ACh receptor and of AChE, which is why it served as a highly appropriate model system for studies directed at these molecules. However, it should be noted that the sedimentation coefficients observed for the enzyme in insects [152], amphibians [246], avians [27], or mammals [150, 229, 357] indicate different molecular weight and/or distinct compositions of catalytic and noncatalytic subunits from those previously reported for the *Torpedo* enzyme.

In addition to AChEs, the above model can also be applied for the molecular forms of BuChE, which have very similar structures and sedi-

mentation coefficients as compared with AChE [215, 337, 342]. A different type of enzyme, described in embryonic chicken cells, contains hybrid tetramers associating two dimers of BuChE with two others composed of AChE subunits [340]. This adds a new dimension to the accepted scheme of molecular forms. It also increases the expected level of complexity of molecular polymorphism to be searched for in this enzyme family.

Apart from the different composition of subunits, the molecular polymorphism generally corresponds to different tissue specifications and subcellular localizations. ChEs are produced in fetal, adult and neoplastic brain tissues [110] including noncholinergic areas such as the cerebellum [109]. The enzymes can be either soluble in the cytoplasm, bound to membrane structures [281], or associated with the extracellular matrix material, all according to their tissue origin. The mechanisms responsible for this complex heterogeneity have not been pursued so far.

It has been demonstrated that the dimeric forms of AChEs contain a short C-terminal domain that may be removed by papain digestion [108] and are generally bound to the plasma membrane [236] or to the endoplasmic reticulum, through a phospholipid link [175] which binds this hydrophobic domain of the protein [129–131, 148] and operates via a specific signal peptide [57]. In addition, the same molecular forms of the enzyme are also present as cytoplasmic soluble proteins. The higher oligomeric forms, that mainly appear as ectoenzymes, may be bound to the plasmatic membrane through a highly hydrophobic 20-kd subunit composed of lipid components. This subunit has been found in the bovine brain [163]. The tetrameric enzyme can also be low salt soluble as in the cerebrospinal fluid, where it is the major AChE form, and in the serum where the soluble BuChE tetramer is predominant. Nothing is known as yet regarding the mode of attachment of BuChE catalytic subunits to solid support.

The asymmetrical forms of AChE are assumed to be associated with the fibrillar molecules of the basal lamina [12] through numerous ionic bonds. This assumption is supported by the observation that these tailed forms become soluble at high ionic strength (e.g., 1 *M* NaCl or 0.5 *M* MgCl) and may be dissociated from their solid support and kept in solution. A small proportion (about 20%) of the asymmetrical form remains associated with lipid membranes even in the presence of salt and may be solubilized by the addition of detergent [131, 303]. Several comprehensive reviews discussing the molecular form heterogeneity in ChEs have appeared [23, 225, 333, 336, 338].

In humans, the ChEs in different tissues also exhibit a high degree of polymorphism, as each expresses a different pattern of molecular forms. Detection of high AChE levels in the amniotic fluid is accepted as a diagnostic in neural tube closure defects [40, 47] or for congenital skin

disorders [34]. In the liver, which is the presumed source of plasma BuChE, the monomeric and dimeric forms of BuChE are detectable and predominant [Soreq et al., unpubl. data]. The external surface of the red cell has long been known to be extremely rich in the AChE dimeric form [254], and to a lesser extent in the monomeric form [255]. Psychiatric stress, among other causes, affects the level of erythrocyte AChE [127].

In the human fetal brain, the main ChE form is a membrane-bound amphiphilic tetrameric AChE [133, 134, 357]. This form represents about 90% of the total activity, excluding the serum activity [271]. It is largely bound to the external surface of the neurons and is assumed to be presynaptic [221]. A small amount of 16 S has been detected, but it represents only 1–2% of the activity [276]. BuChE [19], mainly as tetramer, has also been demonstrated [357]. Preferential transcription of AChE mRNA over BuChE mRNA transcripts was found in fetal human cholinergic neurons, but not in noncholinergic areas such as the developing cerebellum [22]. The cerebrospinal fluid is very rich in soluble 10 S tetrameric AChE, which is probably secreted by the neurons [70, 308].

AChE activity in the cerebrospinal fluid is subject to drastic decreases in Down's syndrome [354] and in Alzheimer's disease [13, 77, 288], similar to the levels of ACh synthesis [137], whereas AChE in the hypophysis decreases following dehydration and is released by stimulation of the pituitary stalk [6]. In the spinal cord the amphiphilic 10 S AChE tetramer is predominant, and its concentration changes under fetal stress conditions [Dreyfus et al., unpubl. data].

In the neurons of the peripheral autonomous nervous system [138], all of the molecular forms of both AChE and BuChE are more or less detectable [103, 165]. This is also the case of the muscle fibre, where, in addition, ChEs are mainly concentrated at the neuromuscular junction [102] and at the myotendinous junctions. In cultured nerve cells, cholinergic properties develop concomitantly with AChE activity [24].

3. Microinjected Xenopus Oocytes as a Heterologous Expression System to Study Cholinesterase Biosynthesis

Several key steps in the biosynthetic pathways responsible for directing the production of the multiple ChE forms have not been elucidated to date. These include posttranscriptional and posttranslational processes, glycosylation and particle segregation patterns along neurites [286] or in other membranous sites. In order to initiate an experimental approach to the molecular mechanisms underlying the biogenesis of this heterogeneous family of enzymes, a full-length cDNA clone coding for human BuChE

[264] was subcloned into the SP6 transcription vector [187]. Synthetic polyadenylated BuChE mRNA was transcribed in vitro and microinjected into *Xenopus* oocytes, where the translation of tissue-extracted ChE mRNA's has previously been demonstrated [311, 312].

Xenopus laevis oocytes have proven to be a valuable in vivo expression system for the production of a variety of biologically active membrane proteins from synthetic and tissue-derived mRNAs [313]. Proteins extensively studied in the oocyte system include the ACh receptor [26, 234], peptide and amino acid neurotransmitter receptors [160], and various channel proteins [85]. In advanced studies, *Xenopus* oocytes have been used in conjunction with site-directed mutagenesis to investigate structure-function relationships in specific polypeptides.

In our hands, the oocytes produced active BuChE displaying enzymatic properties characteristic of the native enzyme [105, 319]. Coinjection of the synthetic BuChE mRNA with total poly(A)$^+$ RNA from brain and muscle was then employed to examine the involvement of additional tissue-specific factors in the assembly and compartmentalization of the enzyme in the oocytes.

4. The Use of Genetic Engineering to Re-Examine the Immunochemical Cross-Reactivity of Anticholinesterase Antibodies

ChEs are rather large proteins, composed of catalytic subunits of ca. 70 kd each. Clearly, the way such polypeptides are folded is bound to play a pivotal role in their biochemical properties. Previous attempts to reveal the molecular origin for the structural heterogeneity of ChEs were based on the elicitation of polyclonal and monoclonal antibodies against minute quantities of highly purified AChE, prepared from the electric organ of *Torpedo* [97, 240, 275], mammalian brain tissue [222, 233, 269] or from red blood cell membranes [45, 119, 309]. The antibodies produced interacted with all of the molecular forms of either AChE or BuChE, respectively. However, antibodies elicited against AChE did not cross-react with BuChE and vice versa [45, 192, 222]. This was generally interpreted to indicate sequence dissimilarities between AChE and BuChE [228]. On the other hand, monoclonal antibodies with significant cross-reactivity have been seen by at least one group [97]. Also, cDNA cloning [228, 264] revealed 53% homology between the amino acid sequence of the human serum enzyme and that of *Torpedo* AChE [294] and, later on, with human AChE [381], including several identical regions of at least 10 successive amino acid residues. This strongly suggests a common ancestral origin for the two genes encoding these enzymes, which could indicate that the lack of

immunological cross-reactivity between AChE and BuChE is not due to a lack of sufficient homology but reflects structural differences. For example, distinct folding patterns of the polypeptide chains could mask homologous regions, or particular glycosylation chains could have the same effect. Another possibility is that the homologous regions are those demonstrating low immunogenicity. To examine these possibilities one would need to elicit antibodies against specific regions of the nascent polypeptide chains that show the greatest homology between various classes of ChE, or which display low immunogenicity.

For this purpose, the N-terminal part of the human BuChE protein, which displays the highest sequence homology to *Torpedo* AChE (amino acid residues 1–198 [see 315]), was produced in bacteria from a recombinant DNA plasmid containing 760 nucleotides from the cloned BuChE cDNA. Antibodies were elicited against this polypeptide and were shown to cross-react with specific molecular forms of both AChE and BuChE from various human tissues.

5. Autoimmune Antibodies to Cholinesterases and Their Clinical Implications

In the neuromuscular junction, various types of conduction defects are known to induce muscle weakness. These include the impaired release of the neurotransmitter ACh, including the Eaton-Lambert syndrome [113], and the disturbed interaction between ACh and the nicotinic ACh receptor, occurring in myasthenia gravis [205]. Severe muscular weakness can also be caused by excessive stimulation, resulting from the accumulation of ACh within the synaptic cleft. This can happen due to inhibition of the neurotransmitter-hydrolyzing enzyme, AChE, as is the case of OP intoxication [181]. Inhibition of AChE in the neuromuscular junction profoundly modifies neuromuscular transmission [305], as has been shown by electrophysiological analyses [193], by studies of the muscle response to nerve stimulation [345], and by observations on spontaneous muscular activity in vivo [118]. In principle, antibodies blocking the activity of AChE in neuromuscular junction should have similar effects.

As mentioned above, both monoclonal and polyclonal antibodies have been raised, for research purposes, against AChE from a long list of species including humans [45, 119, 222]. In many cases, such antibodies presented a strong cross-species and form homology. For example, antibodies raised against both human erythrocyte AChE and *Torpedo* electric organ AChE interact with recombinant human BuChE peptides [104]. However, there are also reports on anti-AChE antibodies which distinguish between two

forms of AChE derived from a single tissue [97]. This is because different forms of ChEs share structurally common domains but also contain distinct regions specific to the particular forms. The spontaneous appearance of anti-AChE antibodies with preferential reactivity for muscle membrane AChE in a patient may serve as an in vivo example for the natural occurrence of this phenomenon and its clinical significance. These antibodies appeared in the serum of a patient manifesting severe diffuse muscular weakness. The findings suggest that an autoimmune response to AChE plays a role in the pathophysiology of neuromuscular dysfunction. However, the problem may not be limited to autoimmune antibodies directed specifically against AChE, but includes reactions against homologous proteins.

Several studies [239, 331] have implied that thyroglobulin (Tg), or an immunogenically Tg-like protein, may be an antigen common to both thyroid and eye orbit. Furthermore, there is evidence that in Graves' disease, the protein eliciting an immunological response in the orbit muscle is not Tg itself [178]. Molecular cloning studies revealed a significant homology between the carboxyl terminal of Tg and the N-terminal half of *Torpedo* AChE [294] and human BuChE [264]. Furthermore, a comparison of their hydropathy profiles [315] and the conservation of cysteine residues involved in disulfide bonds [209, 217] suggest that the two proteins may assume a similar tertiary structure and may share common stereoepitopes. It has been proposed that this homology may explain some pathological symptoms observed in Graves' ophthalmopathy [212, 213], including the lymphocytic infiltration seen in the extraocular muscles. To test this hypothesis, the possibility of cross-reactivity between antibodies to Tg and ChE was investigated. For this purpose, IgGs from patients suffering from Graves' ophthalmopathy were interacted with polyclonal antibodies to both proteins in dot blots and protein electrophoretic blots in which the N-terminal part of recombinant human BuChE, which displays particularly high homology to Tg, was compared with the conventionally prepared human Tg. Furthermore, in situ studies have been performed to determine whether patients' IgGs would bind to end-plate regions of muscle, which are rich in ChE activity, as demonstrated cytochemically. The results are indicative of a causal relationship in this pathological state as well, further extending the autoimmune responses against ChEs.

6. Expression of Cholinesterase Genes in Human Oocytes Examined by in situ Hybridization

The involvement of cholinergic mechanisms in oocyte growth and maturation [115, 313] and sperm-egg interaction [272] has been a subject of