Instituto Juan March
de Estudios e Investigaciones

131 CENTRO DE REUNIONES INTERNACIONALES SOBRE BIOLOGÍA

Workshop on

Genomic vs Non-Genomic Steroid Actions: Encountered or Unified Views

Organized by

M. G. Parker and M. A. Valverde

J.-F. Arnal
F. Auricchio
R. Boland
L. M. García-Segura
G. L. Greene
J. A. Gustafsson
R. A. Hess
M. J. Kelly
F. L. Moore
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M. G. Parker
D. Pearce
G. Schütz
E. Stefani
M. A. Valverde
M. Wehling
M. Whitaker

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Instituto Juan March (Madrid)

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Introduction
M. G. Parker and M. A. Valverde

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Steroid hormones have been known for decades to be involved in various physiological responses. Their interaction with receptors of the steroid/thyroid family, best known as transcriptional activators, have captivated the attention of investigators since the early days of the study of their mechanism of action. More recently, over the last few years, we have witnessed a scientific revolution that has led to the identification of many novel mechanisms of action for these molecules.

The classical view of steroid action proposes that steroids modulate gene expression via their nuclear receptors. These receptors act as transcription factors regulating transcription following the recognition of hormone response elements at the DNA. The ligand-dependent modulation of transcription results in changes in protein synthesis with a time delay, typically, in the range of hours.

On the other hand, the novel mechanisms of action of steroids result in biological responses with typical characteristics as follows: i) rapid time-course (from seconds to minutes) so that the primary effect is too fast to be compatible with either RNA synthesis or protein translation; ii) they can be either dependent or independent to the presence of classical steroid receptors; iii) the extracellular membrane-delimited primary effect might be achieved by steroid conjugated to membrane-impermeable molecules and iv) the mechanism of action generally employs the generation of intracellular signals.

Interestingly, the novel findings (termed "non-genomic", or "alternative pathways") do not integrate nicely into the well established field of the genomic action of steroids (also known as "classic pathway"). The reasons for this lack of communication are difficult to understand and beyond the aim of this short introductory document. However, the fact is that as a result of this posture, no exchange of information, no constructive discussion between these 'encountered' worlds has taken place for far too long.

When planning the organization of the meeting we thought that the main interest would reside in bringing together views from both parties, "genomic *vs* non-genomic". With this purpose in mind we faced the difficult task of selecting specific areas of interest in which both classic and alternative views could be represented. Five areas were chosen: the functional localization of steroid receptors within the cell, intracellular signalling generated in response to steroids as well as the cross-talk between different signalling cascades involved in steroid function, the regulation of membrane excitability by controlling either the expression or activity of plasma membrane ion channels and analysis of the complex phenotype of different steroid receptor knock-out models.

The experiment offered a very positive response. The amount of novel information presented in the talks and the posters as well as the discussion they inspired was amazing. A constructive exchange of information between both parties took place and it was the general view that the existence of one mechanism in a particular cell is not exclusive. In this way, steroids can elicit diverse cellular effects that might depend on their concentration and their primary target. Hopefully, this unified view of steroid actions will soon be feasible and will integrate their different mechanisms of action. In the mean time, we are all expectant about the advances taking place in the steroid actions´ field, especially those related to the molecular identification of new targets, the cross-talk between different signalling cascades and the unravelling of the very complex interaction of nuclear receptors with coactivators and repressors within the cell nucleus.

Malcolm G. Parker and Miguel A. Valverde

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Session 1: Steroid receptors: now in the cytosol, now in the membrane
Chairs: Jan-Åke Gustafsson and Frank L. Moore

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ERα and ERβ: Complexities of ligand recognition and its consequences

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Estrogens and SERMs (selective estrogen receptor modulators) regulate diverse cellular activities via one or both of two known estrogen receptor subtypes (ERα and ERβ) in hormone responsive tissues and cancers. Liganded ERs can interact with a complex mix of coactivators, corepressors and other signaling molecules that differ in expression and importance from tissue to tissue. In addition, different SERMs may alter the affinity and/or selectivity of one or both ERs for these coregulators, allowing for tissue selective responses. Recently, insight into the molecular basis of estrogen agonism and antagonism has been revealed by the crystal structures of ERα and ERβ ligand binding domains (LBDs) complexed with several ligands, including estradiol (E2), diethylstilbestrol (DES), raloxifene (RAL), 4-hydroxytamoxifen (OHT), and the phytoestrogen genistein (GEN). For agonists like DES, inclusion of a peptide derived from an essential LXXLL interaction motif (NR box) found in several related p160 nuclear receptor transcriptional co-activators has helped define the AF-2/co-activator interface on ER. Although agonists and antagonists bind at the same site within the core of the LBD, each induces distinct conformations in the transactivation domain (AF-2) of the LBD, especially in the positioning of helix 12, providing structural evidence for multiple mechanisms of selective antagonism in the nuclear receptor family. Interestingly, the OHT/RAL and DES/E2 structures collectively reveal and define a multipurpose docking site on ERα that can accommodate either helix 12 or one of several coregulators. In addition, a comparison of the two structures reveals that there are at least two distinct mechanisms by which structural features of OHT promote an inhibitory conformation of helix 12. Helix 12 positioning is determined both by steric considerations, such as the presence of an extended side chain in the ligand, and by local structural distortions in and around the ligand binding pocket. Thus, one would predict that effective estrogen antagonists do not necessarily require bulky or extended side chains.

Ligands that act differentially on ERα and ERβ are potentially valuable both as tools to dissect the biological roles of the two ERs and as novel therapeutics with pharmacological properties distinct from existing drugs. As part of a search for ER subtype-selective ligands, the synthetic compound, R,R-5,11-*cis*-diethyl-5,6,11,12-tetrahydrochrysene-2,8-diol (R,R-THC), was identified as a selective estrogen agonist when bound to ERα and as an antagonist when bound to ERβ. To better understand this selective behavior, the crystallographic structures of human ERα and ERβ ligand binding domains (LBDs) complexed with R,R-THC were solved and refined, suggesting mechanisms by which this compound can act as an ERα agonist and as an ERβ antagonist. Consistent with the prediction that bulky/extended side chains are not essential for antagonist behavior, R,R-THC antagonizes ERβ in a manner very different from OHT and RAL. The positioning of the side chains of OHT, RAL, and ICI directly or "actively" preclude the agonist-bound conformation of helix 12 by steric hindrance. Hence, we define their common mechanism of action as "active antagonism". R,R-THC does not have a bulky side chain and in its complex with ERβ, helix 12 is not sterically precluded from adopting the agonist-bound conformation as it is in the other antagonist complexes.

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Instead, R,R-THC antagonizes ERβ by stabilizing key ligand binding pocket residues in noninteracting conformations, and disfavoring the equilibrium to the agonist-bound conformation of helix 12. Thus, we term the mechanism of antagonism of R,R-THC as "passive antagonism". The passive antagonism mechanism, as revealed here through direct comparison of the two R,R-THC-ER LBD complexes, suggests a new approach to achieving NR antagonism. Compounds could be designed to selectively stabilize the inactive conformations of certain NR subtypes and the active conformations of others. Such ligands may exert novel biological and therapeutic effects. Ultimately, this information must be coupled with information about expression patterns for both ER subtypes as well as essential coregulatory proteins or intersecting signal pathways to predict the behavior of a given SERM in a responsive tissue.

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The nuclear-receptor interacting proteins

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Estrogen signalling may take place and/or be modulated through a wide variety of coregulators. Some of these appear to constitute subunits of preexisting protein complexes or protein machines like the SRC/CBP or TRAP complexes whereas others seem to be recruited to the estrogen receptors as monomers, such as SHP and DAX-1. A third category is examplified by RAP250 where information is still lacking regarding its possible protein neighbours in its immediate environment. The two estrogen receptors, ERalfa and ERbeta, appear to differ in their preference for protein coregulators as is also indicated from the different sedimentation properties of the two receptors on sucrose density gradients in low salt. A deeper understanding of ERalfa and ERbeta specific interaction with downstream mediators of hormone signalling is essential in understanding the molecular mechanisms underlying the different biological action profiles of the two estrogen receptors. Such knowledge is essential in defining putative new targets for new generation drugs specifically modulating ERalfa and ERbeta action, respectively.

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Searching for the molecular structure of the amphibian corticosterone membrane receptor

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The neuroendocrine mechanisms that control rapid behavioral responses to acute stress are highly conserved among vertebrates and involve the peptides and steroid hormones in the hypothalamic-pituitary-adrenal axis (HPA), plus the peptides in the vasopressin and opioid families. Behavioral studies in an amphibian model, the roughskin newt (*Taricha granulosa*), demonstrate that acute stress triggers the secretion of corticosterone and that the elevated corticosterone concentration causes a rapid suppression of reproductive behaviors. This behavioral response to corticosterone is too rapid (occurring within a few minutes) to be easily explained by the classic genomic model for steroid hormones and, instead, works through a non-genomic pathway that involves a membrane-associated corticosteroid receptor (mCR) in the G-protein coupled receptor superfamily (Orchinik et al., 1991; 1992). Partial purification of the solubilized mCR using sequential chromatography schemes revealed a putative receptor protein with an apparent mass of 63 kDa (Evans et al., 2000a). This finding was confirmed using two independent systems--a differential-display CORT-Sepharose affinity chromatography that visualized the protein on 2-D SDS-PAGE and a photoaffinity-labeling system that visualized the protein with western blot methodology and anti-CORT antiserum. Pharmacological characterization of mCR found that, of the many steroids tested, the [^{3}H]-CORT binding site is highly selective for corticosterone and cortisol (Orchinik et al. 1991). Recent competition binding assays revealed that the [^{3}H]-CORT binding site also recognizes a subset of kappa-selective ligands and that these ligands interact directly (not allosterically) by competing for the same binding pocket (Evans et al. 2000b). These binding data are consistent with behavioral and physiological studies showing that corticosterone and specific kappa ligands trigger similar responses. To our working hypothesis that this mCR is structurally related to a kappa opioid-like receptor, we are currently cloning and pharmacologically characterizing opioid-like receptors in newt brains.

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Structure and function of human sex hormone-binding globulin (SHBG)

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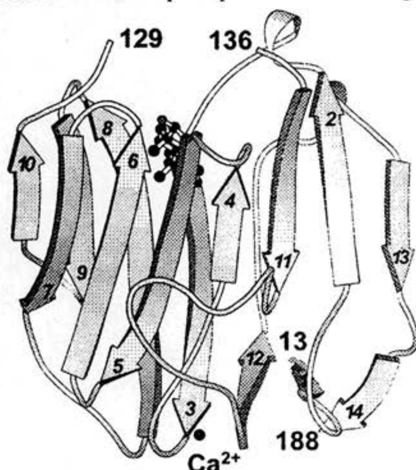
Human sex hormone-binding globulin (SHBG) is a highly specific plasma transport protein for sex steroids. It is a homodimeric glycoprotein and each monomer consists of a tandem repeat of laminin G-like (LG) domains. In a first step we determined the crystal structure of the N-terminal LG domain of SHBG in complex with the steroid 5α-dihydrotestosterone (DHT) and revealed both the general architecture of the LG-domain fold and in particular the structural determinants of the steroid-binding site of SHBG. The LG domain of SHBG consists of a β-sandwich formed by two seven-stranded β-sheets. The steroid-binding site is located at the rim of the β-sandwich and the steroid intercalates in between the two β-sheets. A comparison with other LG domain-containing proteins shows that LG domains appear to have a preferential ligand interaction site at the rim of the β-sheet sandwich rather than on the concave site of the β-sandwich, as observed for the structurally related legume lectins.

Our recent studies focused on questions regarding the steroid-binding specificity of SHBG. Thus, we observed that the affinity for estradiol is reduced in the presence of 0.1-1 mM Zn2+, and chemically related metal ions (Cd2+ and Hg2+) have similar but less pronounced effects. Crystal soaking experiments showed that binding of zinc in immediate proximity to the steroid-binding site of SHBG has two distinct effects. First, zinc binding reorients Asp65, which in the absence of zinc contacts the steroid, and secondly, zinc binding induces disorder in a loop segment, which in the absence of zinc covers the steroid-binding site.

The crystal structures of SHBG in complex with various steroids revealed unexpected differences in the binding modes of androgens and estrogens to SHBG. These studies also showed how the synthetic contraceptive Norgestrel is accommodated in the binding-site and that the beta orientation of the OH-group at position 17 is not a prerequisite for the binding of androgens to SHBG. Combining this data with mutagenesis experiments in which residues lining the steroid-binding pocket have been substituted allows for an in depth description of the steroid-binding determinants of SHBG.

The studies on the structure and function of SHBG presented here are in close collaboration with the group of Geoffrey Hammond from the University of Western Ontario in London Ontario, Canada.



Crystal structure of the N-terminal domain of SHBG in complex with 5-α-dihydrotestosterone

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Rapid effects of retinoic acid on CREB and ERK phosphorylation in Protein Kinase A-deficient PC12 cells.

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Retinoic acid (RA) causes differentiation with neurite extension in A126-1B2 cells, a PC12 cell subclone deficient in protein kinase A (PKA) activity. A central process in differentiation of PC12 cells by neurotrophins such as nerve growth factor (NGF), is the activation by phosphorylation of the transcription factor CREB (cyclic AMP response element binding protein), through activation of the mitogen activated protein kinase (ERK) cascade. CREB phosphorylation induces transcriptional activation of target genes that contain binding sites for this factor (CREs) in their regulatory regions. Treatment of A126-1B2 cells with RA induces a rapid phosphorylation of CREB at Ser133, compatible with an extragenomic effect of this ligand. Phosphorylation is maximal at 10 min and remains elevated even after 8 hours of incubation with RA. TTNPB, a ligand specific for the RA receptors RARs, also induces CREB phosphorylation in A126-1B2 cells, indicating a role for these receptors in this process. A126-1B2 cells express higher levels of RARs than parental PC12 cells where RA causes neither differentiation nor CREB phosphorylation. RA increases transcriptional activity of GAL-CREB in transfection assays, and mutation of Ser133 abolishes this stimulation. In addition, incubation with RA increases the activitity of the c-*fos* promoter, as well as other CRE-containing promoters in A126-1B2 cells. Activation of the c-*fos* promoter is abolished by a dominant-negative mutant of CREB, showing that CREB phosphorylation plays a key role in RA-mediated stimulation of this promoter. The effect of RA on CREB phosphorylation is similar to that produced by NGF and the combination of both factors did not cause a further increase, suggesting that they could use similar pathways to stimulate CREB phosphorylation. Indeed, RA also causes a strong activation of ERK 1 and 2 in A126-1B2 cells. ERK phosphorylation is transient, since it is observed after 3 min of incubation, but it dissapears after 60 min. UO126 inhibits RA-mediated CREB phosphorylation and c-fos promoter activation, showing that ERKs are required for these effects of the retinoid.

In summary, our data demonstrate that RA stimulates signalling pathways and expression of early genes involved in neuronal differentiation by a non genomic mechanism that does not appear to involve binding of its receptors to RA response elements.

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Session 2: Steroids and intracellular calcium
Chairs: Michael Whitaker and Hans Oberleithner

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Calcium signalling mechanisms of the cell division cycle

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A single calcium spike occurs just before first mitosis in sea urchin embryos; a further spike occurs once embryos have entered mitosis, just before the onset of chromatid separation. If the calcium transients are severally blocked using calcium chelators or $InsP_3$ receptor antagonists, entry into mitosis and chromatid disjunction are prevented. Blocked embryos can be released by generating an exogenous calcium spike using caged calcium or caged $InsP_3$ (Wilding et al., 1996; Groigno & Whitaker, 1998).

Entry into and exit from mitosis are also prevented by microinjection of the protein SH2 domain from a mammalian PDGF receptor that is thought to prevent activation of PLCγ and by treatment of early embryos with lithium. Intracellular $InsP_3$ concentrations increase just before mitosis and just prior to anaphase onset (Shearer et al., 1999).These data indicate that the phosphoinositide messenger pathway generates calcium signals that are necessary both for entry into mitosis and for chromatid disjunction during the first cell division in sea urchin embryos.

TA-calmodulin is a calmodulin activation probe that permits the imaging of calmodulin activation in the confocal microscope. During mitosis, calmodulin activation is first seen close to the nucleus in sea urchin embryos just before breakdown of the nuclear envelope and entry into mitosis and again at the spindle poles just before chromatid disjunction and anaphase onset. Microinjecting a myosin light chain kinase-based inhibitory peptide prevents the events of mitosis (Török et al., 1998; Whitaker, 2000). These observations demonstrate that local activation of calmodulin around the nucleus and mitotic spindle control the events of mitosis, indicating that one major target of the mitotic calcium signals is calmodulin.

The first 14 cell divisions of the Drosophila embryos occur in the same cytoplasm. Nuclei initially divide deep within the embryo, but move to the surface by cycle 10, where they are readily observed in the living embryo. Mitosis is quasi-synchronous: each cell cycle stage is most advanced at the poles and less advanced at the equator of the embryo. This gives the appearance of two waves of anaphase onset that begin at the poles and sweep towards the equator.Mitoses become asynchronous after cycle 14, when the embryo cellularizes.

We have measured cytoplasmic calcium concentrations in Drosophila embryos during cycles 10-14, using confocal microscopy and calcium green/rhodamine dextran ratio imaging.Calcium waves pass along the embryo from pole to equator, where they annihilate. Simultaneous measurement of calcium and visualization of microtubules or histone using fluorescent tags show that the waves are in synchrony with the cell cycle: calcium is highest in interphase.Local increases in calcium are seen around the mitotic spindle just before anaphase. Glancing cortical confocal sections show that the calcium levels are locally inhomogeneous.

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The pattern of calcium release mirrors the pattern of the distribution of endoplasmic reticulum, as seen using a carbocyanine dye in living embryos. Tagged fluorescent actin shows a similar dynamic distribution.

Microinjection of heparin, an $InsP_3$ antagonist, abolishes the calcium wave and arrests the cell cycle in both interphase and mitosis. A very similar result is seen after microinjection of recombinant PH-domain constructs that interfere with phosphoinositide signalling.
These data from Drosophila suggest that calcium signals control cell cycle progression in Drosophila, just as they do in early sea urchin embryos.

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Role of calcium in aldosterone-mediated signaling through nuclear pores

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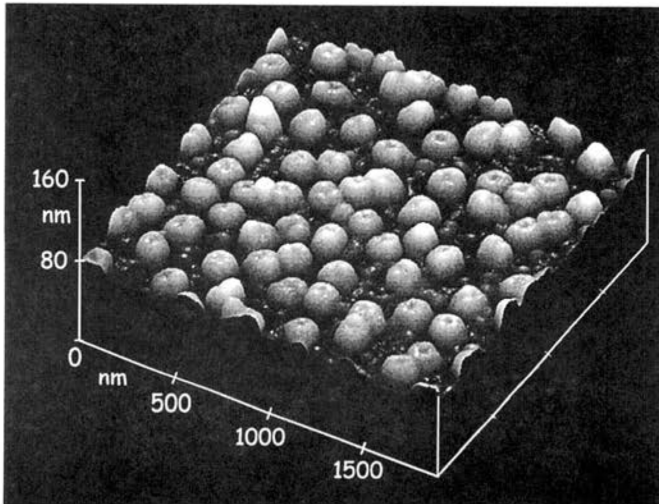
First approach:

Initiation of transcription is an early step in steroid hormone action. We investigated with atomic force microscopy (AFM). DNA fluorescence imaging and electrical techniques the role of nuclear pore complexes (NPCs) in mediating signal transduction of the mineralocorticoid hormone aldosterone from the extracellular space into the cell nucleus. With AFM we imaged single NPCs of isolated nuclear envelopes under native conditions. We observed that individual NPCs contract in response to a Ca2+ signal known to occur in seconds after aldosterone exposure. In living kidney cells aldosterone leads in seconds to the contraction of the whole nucleus measured by DNA-fluorescence imaging. Nuclear contraction was elicited at similar time scale and to similar extent by bradykinin, a peptide hormone known to mobilize Ca2+ from internal stores and by ionomycin, a Ca2+ ionophore known to directly increase intracellular Ca2+.

First Conclusion:

ontraction of the cell nucleus is explained by the individual contraction of calcium-sensitive NPCs that occur in high density in the nuclear envelope (1).

Figure 1:



AFM image of nuclear pore complexes (NPCs) in Xenopus laevis oocyte. NPCs in the nuclear envelope represent the channel pathways for ions and macromolecules between cytosol and nucleoplasm.

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Second approach:

Combining electrophysiology and AFM we describe the route by which macromolecules enter and exit the nucleus across the nuclear envelope of *Xenopus laevis* oocytes. Individual stage VI oocytes were microinjected with 50 fmol aldosterone and then enucleated 2 to 30 minutes post injection. Nuclear envelope electrical resistance (NEER) was then measured in the isolated nuclei by using the nuclear hourglass technique (2, 3). We observed three NEER stages: an early NEER peak two minutes after injection, a sustained NEER depression after 5 to 15 minutes and a final late NEER peak 20 minutes after injection. Since NEER reflects the passive electrical permeability of nuclear pore complexes (NPCs) we investigated with AFM the molecular conformation of individual NPCs in the nuclear envelope. At the early NEER peak (2 minutes after aldosterone) we observed 100 kD molecules (flags) attached to the NPC surface. At the sustained NEER depression (5 to 15 minutes after aldosterone) NPCs were found free of flags. At the late NEER peak (20 minutes after aldosterone) 800 kD molecules (plugs) were detected in the NPC central channels. Ribonuclease treatment eliminated the late NEER peak. Co-injection of aldosterone (50 fmol) and its competitive inhibitor spironolactone (500 fmol) prevented the NEER changes as well as flag & plug formation.

Second conclusion:

Flags represent aldosterone receptors/transcription factors translocated into the cell nucleus while plugs represent ribonucleoproteins carrying aldosterone induced mRNA from the nucleoplasm to the cytoplasm.

Perspective:

We currently test whether free intracellular calcium is a prerequisite for initiating the genomic route of aldosterone action.

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17β-Estradiol actions on pancreatic β-cells via a plasma membrane receptor unrelated to ERα and ERβ

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In pancreatic β-cells, 17β-estradiol actions are mediated via a membrane receptor unrelated to nERs, as manifested by immunohistochemistry and binding experiments (Nadal et al, 1998; Nadal et al 2000). Neither the anti-ERα nor the anti-ERβ antibodies stained the membrane of nonpermeabilised β-cells, despite the existence of oestrogen binding sites. This novel pmER is not blocked by the antiestrogen ICI 182,780 and its binding site is shared by oestrogens, catecholoestrogens, xenoestrogens and catecholamines. It activates a guanylate cyclase, inducing an increase of cGMP and an activation of PKG, which leads to a regulation of K_{ATP} channels (Ropero et al, 1999). As a result, $[Ca^{2+}]_i$ is increased triggering an enhanced insulin secretion and CREB phosphorylation.

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Regulation of calcium signalling pathways in skeletal muscle cells by 1,25(OH)2-vitamin D3

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The secosteroid hormone 1,25-dihydroxy-vitamin D3 [1,25(OH)2D3] plays a role in the regulation of skeletal muscle intracellular Ca2+ homeostasis, thus underlying the alterations in contractility observed in states of 1,25(OH)2D3 deficit (1). As in other target cells, 1,25(OH)2D3 elicits long-term and short-term responses in muscle which involve genomic and non-genomic mode of actions, respectively. In the first mechanism, through nuclear vitamin D receptor (VDR)-mediated gene transcription the hormone induces the synthesis of calcium binding proteins (2). 1,25(OH)2D3 also exerts rapid effects in skeletal muscle initiated in the cell surface, followed by the stimulation of second messenger systems which transmit the signal to the cytoplasm. This novel mode of action involves the activation of Ca2+ entry through voltage-dependent channels or VDCC (2,3) by G protein-mediated modulation of the adenylyl cyclase/cAMP/PKA (4) and fosfolipase C/DAG + IP3/PKC α (5,6) pathways. 1,25(OH)2D3 also stimulates the release of Ca2+ from intracellular stores (7) and the capacitative influx of the cation through SOC ("Store Operated Calcium") channels (8). Data have been obtained involving tyrosine kinases (TKs) and the VDR in hormone regulation of the Ca2+ messenger system by mediating the stimulation of SOC (TRP) channels. Congruent with these results, 1,25(OH)2D3 induces a rapid translocation of the VDR from the nucleus to the plasma membrane, process which is blocked by TK inhibitors (9). Of mechanistic relevance, molecular and immunochemical studies and the application of oligonucleotide antisense technology in conjunction with microspectrofluorimetric analysis have provided evidences on the participation of signaling supramolecular structures integrated by TRP proteins, calmodulin, TKs and the VDR in the modulation of SOC influx by 1,25(OH)2D3 (10-12). Moreover, these studies suggest that the PDZ domain-containing INAD protein may play a role serving as scaffold for assembling the hormone signaling molecules (13).

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Session 3: Steroids and ion channels
Chairs: Enrico Stefani and Martin J. Kelly

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Hormonal regulation of ion channels

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We are investigating the role of sex hormones on the expression levels of MaxiK and Kv.4.3 channels in rat myometrium. Using site directed antibodies, RT-PCR and RNAse protection assay (RPA), we detected MaxiK and Kv4.3 channel protein and transcripts in myometrium. Western blot analysis with anti-MaxiK anti-Kv4.3 antibody recognizes a protein with the expected molecular sizes (110 and 68 kDa, respectively) in myometrium membranes. MaxiK channels were equally localized in the circular and longitudinal smooth muscle layer, while Kv4.3 channels were mainly localized in the circular smooth muscle. The protein-antibody interactions were fully inhibited when the antibody was pre-adsorbed with the corresponding antigen (10 μg/ml). Since K^+ currents may regulate myometrial rhythmic contraction and tone, we determined their relative expression at different stages of pregnancy.

MaixK and Kv4.3 channel signals were especially strong in non-pregnant myometrium and at early pregnancy. On the other hand, in late pregnancy the MaxiK and Kv4.3 signals were dramatically reduced. The MaxiK and Kv4.3 protein expression level correlates with mRNA levels quantified with RPA. This correlation suggests a hormonal control at the transcription level. To investigate which sex hormone was involved in this regulation we treated ovariectomized rats with β-estradiol (E) and progesterone(P) and measured MaxiK and Kv4.3 expression levels with Western blot analysis. Rats received a 4-day treatment (2 injections/day) with sesame oil (control), 0.6 mg progesterone (P), 10 μg β-estradiol (E) or P+E. In myometrium, control and P treated rats showed no significant difference in MaxiK and Kv4.3 protein levels, while in E and P+E treated rats, a significant reduction of MaxiK and Kv4.3 labeling was observed. Despite of the large changes observed in myometrium, the hormonal treatment did not produce any variation in brain and heart Kv4.3. These results show that β-estradiol induces a tissue specific downregulation of MaxiK and Kv4.3 protein levels in myometrium. This reduction in Kv4.3 channels would enhance myometrium excitability. The decline of K channels later in pregnancy possibly induced by the rise of estrogens may facilitate uterine contraction needed for parturition. Supported by NIH grants. LT is an Established Investigator of AHA.

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Modulation of vascular smooth muscle ion channels by estrogens

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Rapid modulation of vascular smooth muscle ion channels by estrogens leads to endothelium-dependent and independent vasodilatation. A key player in the control of vascular smooth muscle tone is the Maxi-K channel. This channel consists of two subunits: a pore forming α subunit and a regulatory β subunit which confers the channel with a higher $Ca2+$ sensitivity. We have recently described the modulation by 17β-estradiol of both native and heterologously expressed Maxi-K channels and found that oestradiol activates the channels through its interaction with the β subunit (Valverde *et al.* 1999).

Xenopus laevis were injected with mRNA encoding the α subunit alone or in combination with mRNA encoding the β subunit. Maxi-K currents were recorded using the patch-clamp technique. The presence of 17β-estradiol in the extracellular side of the membrane elicited an increase in the Maxi-K currents recorded from $\alpha\beta$ expressing patches but not in those expressing α subunit alone. 17β-Estradiol conjugated to albumin, a membrane-impenetrable carrier, activated Maxi-K currents only when added extracellularly. Fluorescence microscopy studies confirmed the interaction of 17β-estradiol (conjugated to albumin and FITC) with the β subunit of Maxi-K channels in a cell line engineered to express one or both channel subunits. We have also evaluate the interaction of estradiol with different Maxi-K β subunits with the aim to unveil the molecular nature of the modulation of Maxi-K by oestrogens.

Estrogen and antiestrogens are also capable of modulating the activity of Maxi chloride channels in vascular smooth muscle cells (Díaz et al. 1999) . The mechanism of action leading to the modulation of Maxi-Cl channels seems to be different from the one use to modulate Maxi-K channels. In the former, cellular signalling is necessary while in the later direct interaction of estrogens with the channel protein is sufficient.

Our results suggest that estrogen and antiestrogens exert different rapid actions on the same cell type, an observation that fits the current view of multiple sites of action for estrogens (Nadal et al. 2001).

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Small conductance calcium-activated potassium channels (SK) mRNA expression: modulation by estrogen

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Small conductance calcium-activated potassium (SK) channels underlie the slow afterhyperpolarization (AHP), which is critical for sculpturing phasic firing in hypothalamic neurosecretory neurons including gonadotropin-releasing hormone (GnRH), dopamine and vasopressin neurons. Molecular cloning has revealed the existence of three distinct SK channels (SK1-3), and we have investigated the distribution and effects estrogen on two of these channels.

Both SK1 and SK3 cDNA fragments were cloned using RT-PCR, and ribonuclease protection assay as well as in situ hybridization analysis revealed that the SK3 channel was the predominant subtype expressed in the guinea pig hypothalamus. Combined in situ hybridization and fluorescence immunocytochemistry ascertained that SK3 mRNA was expressed in GnRH, dopamine, and vasopressin neurons, and all of these neurons exhibited an apamin-sensitive AHP current as measured with whole-cell recording. Using quantitative RPA, the rank order of SK3

mRNA expression was septum ^{3} midbrain > rostral thalamus ^{3}rostral basal hypothalamus ^{3} caudal thalamus ^{3} preoptic area >> caudal basal hypothalamus ^{3}hippocampus. Moreover, E2 treatment significantly increased SK3 mRNA levels in the rostral basal hypothalamus during the negative feedback phase (low LH levels) ($p<0.05$; $n=6$), but not during the positive feedback phase (high LH levels) of the ovulatory cycle. Therefore, these findings suggest that estrogen increases the mRNA expression of the SK3 channel, which may represent a mechanism by which estrogen limits hypothalamic neuronal firing during negative feedback of the female ovulatory cycle.

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Epithelial Na+ channel regulated by aldosterone-induced protein, SGK1

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Sodium transport in epithelia is regulated by a variety of hormonal and non-hormonal factors including mineralocorticoids, insulin, shear stress and osmotic pressure. In mammals, the mineralocorticoid aldosterone is the principal regulator of sodium homeostasis, and hence is central to the control of extracellular fluid volume and blood pressure. Aldosterone acts through a member of the nuclear receptor superfamily, the mineralocorticoid receptor (MR), to control the transcriptional activity of specific target genes. Although a role for non-genomic factors in regulating epithelial ion transport has not been ruled out, it is clear that genomic mechanisms are essential.

Several candidate target genes have been suggested as mediators of mineralocorticoid action in epithelia, including genes encoding the transporters themselves, genes encoding enzymes of intermediary metabolism and genes encoding regulatory proteins, such as protein kinases. One such regulatory protein is SGK1 (serum and glucocorticoid-regulated kinase isoform 1), identified using a PCR-based subtraction method as a mineralocorticoid-regulated gene in kidney tubule cells. SGK1 is a close relative of PKB/Akt, a mediator of insulin effects on metabolism. MR increases SGK1 gene transcription and SGK1 protein is then activated through a phosphatidylinositol-3-kinase (PI3K)-dependent phosphorylation event to a form that strongly stimulates the epithelial sodium channel (ENaC). ENaC constitutes the rate limiting step for Na+ transport in aldosterone-responsive epithelia and is the principal early target of aldosterone action. Consistent with its functional role in mediating the early effects of aldosterone, SGK1 strongly stimulates ENaC plasma membrane localization.

Recent evidence has firmly established SGK1 as a key mediator of mineralocorticoid effects. First, an SGK1 knockout mouse has a sodium wasting phenotype consistent with the proposed role of SGK1 as a stimulator of sodium retention. Second, blocking SGK1 induction by aldosterone in cultured cells leads to a decrease in sodium transport. Finally, expression of exogenous SGK1 in cultured cells stimulates sodium transport. The latter result is consistent with the idea that transcriptional mechanisms alone are sufficient to mediate steroid effects on ion transport in epithelia.

SGK1 gene transcription is highly dynamic in that it is rapidly and strongly stimulated by mineralocorticoids but then soon begins to return toward baseline levels, a process we term deinduction. The rapid deinduction of SGK1 occurs in cultured kidney cells, as well as in rat kidney, and is not dependent on global inactivation of the receptor function or to metabolism of hormone to an inactive form. Rather the effect is specific to the SGK1 gene itself, reflecting an inactivation process of unknown mechanism. Other regulators of SGK1 gene transcription,

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including osmotic shock, also have transient effects. In this sense, SGK1 is a classic immediate early gene, in the manner of cFos.

The last several years have seen marked progress in understanding the mechanisms underlying hormone-regulated Na+ transport. As the molecular components of the regulatory machinery have been identified, a fuller mechanistic understanding of these processes has become accessible. Currently, the hormone receptors, the ion transporters themselves (including ENaC subunits, Na/K-ATPase, potassium channels etc.), and regulatory proteins have been identified. SGK1 represents one such regulatory protein. It sits strategically at a point in the signal transduction process that allows it to integrate the effects of several regulatory inputs, including steroid hormones, osmotic shock and non-steroid hormones such as insulin and TGFß. Some of these factors, such as mineralocorticoids influence primarily SGK1 expression level, others, such as insulin, influence primarily its activity. Interestingly, osmotic shock, an ancient regulator of cell ion transport, appears to regulate both the expression level and activity of SGK1.

The dual regulation of SGK1-- its abundance through a transcriptional mechanism and its activity through a PI3K-dependent pathway-- provides a mechanism for integrating multiple different inputs that impact on Na+ influx into epithelial cells, particularly those of aldosterone, insulin and osmolarity (through effects on cell volume). The ability of SGK1 to respond to various inputs may have played a central role in evolution, allowing early single cell organisms to respond to conditions that alter cell volume, and later allowing vertebrates to make the transition to freshwater and then to land. In humans, SGK1 might be implicated in medical conditions such as the insulin resistance syndrome, hypertension and congestive heart failure.

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Estrogen modulation of potassium channel activity in the CNS

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Voltage-gated and ligand-gated K+ channels are important regulators of cellular excitability. In the CNS, they are responsible for repolarization of the action potential and modulating the firing frequency of neurons. We have focused on the role of these channels in hypothalamic neurons that control reproduction, stress and motivated behaviors such as feeding. We have targeted gonadotropin-releasing hormone (GnRH) neurons, proopiomelanocortin (POMC) and -aminobutyric acid (GABA) neurons. We have examined the modulation by 17 -estradiol (E2) of the coupling of various receptor systems to G protein-gated, inwardly-rectifying K+ (GIRK) channels and small conductance, Ca2+-activated K+ (SK) channels. In an ovariectomized female, E2 rapidly uncouples μ-opioid receptors from GIRK channels in -endorphin neurons, as manifest by a reduction in the potency of μ-opioid receptor agonists to hyperpolarize these cells. This effect is blocked by inhibitors of protein kinase A and protein kinase C . A selective E2 receptor is involved in that equimolar concentrations of 17 -estradiol are ineffective, and the anti-estrogen ICI 164,384 blocks these actions of estrogen with sub-nanomolar affinity. E2 also uncouples GABAB receptors from the same population of GIRK channels which are coupled to μ-opioid receptors. At 24 hours after steroid administration, the GABAB/GIRK channel uncoupling observed in GABAergic neurons of the preoptic area is associated with reduced agonist efficacy.

Another class of K+ channels, the small conductance, Ca2+ -activated K+ channels, are responsible for the afterhyperpolarization (AHP) following action potential firing in a number of CNS neurons. Three SK channel genes, which underlie the expression of the AHP current in CNS neurons, have been cloned, and we have found that the SK3 mRNA is highly expressed in hypothalamic neurons and is up-regulated by E2. Both 1 and -noradrenergic agonists potently inhibit the AHP current in preoptic neurons thereby increasing the firing rate. E2 enhances the efficacy of 1-adrenergic receptor agonists to inhibit AHP currents in preoptic GABAergic neurons, and does so in both a rapid and sustained fashion. Finally, we have observed a direct, steroid-induced hyperpolarization of GnRH neurons via activation of inwardly-rectifying K+ channels. Therefore, E2 can activate kinase pathways (PKC and PKA) to modulate transmitter coupling to GIRK and SK channels or directly activate K+ channels in other (GnRH) hypothalamic neurons, all of which are critical for controlling homeostasis and behavior.

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Session 4: Cross-talk between different signalling pathways
Chairs: Martin Wehling and Ferdinando Auricchio

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Putative steroid membrane receptors: aldosterone and progesterone membrane receptors

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According to the traditional model steroid hormones bind to intracellular receptors and subsequently modulate transcription and protein synthesis, thus triggering genomic events finally responsible for delayed effects. Based upon similarities in molecular structure, specific receptors for steroids, vitamin D_3 derivatives, thyroid hormone, retinoids and a vanity of orphan receptors are considered to represent a superfamily of steroid receptors. In addition, very rapid effects of steroids mainly affecting intracellular signaling have been widely recognized which are clearly incompatible with the genomic model. These rapid, nongenomic steroid actions are likely to be transmitted via specific membrane receptors. Evidences for nongenomic steroid effects and distinct receptors involved are now presented for all steroid groups including related compounds like vitamin D_3 and thyroid hormones. The physiological and clinical relevance of these rapid effects is still largely unclear, but their existence in vivo has been clearly shown in various settings including human studies. Drugs that specifically affect nongenomic steroid action may find applications in various clinical areas such as cardiovascular and central nervous disorders, electrolyte homeostasis and infertility. In addition to a short description of genomic steroid action, this review pays particular attention to the current knowledge and important results on the mechanisms of nongenomic steroid action. The modes of action are discussed in relation to their potential physiological or pathophysiological relevance and with regard to a cross-talk between genomic and non-genomic responses.

Prominent examples of nongenomic steroid action are rapid aldosterone effects in lymphocytes and vascular smooth muscle cells, vitamin D3 effects in epithelial cells, progesterone effects in human sperm, neurosteroid action on neuronal structures and vascular effects of estrogens. Mechanisms of action are being studied with regard to signal perception and transduction involved, and for various steroids including aldosterone a patchy sketch of a membrane receptor/second messenger cascade shows up in the mist being not essentially dissimilar to cascades involved in catecholamine or peptide hormone action. Aside nonclassical membrane receptors with a high affinity for a particular steroid, these effects appear to variably involve phospholipase C, phosphoinositide turnover, intracellular pH and calcium, protein kinase C and tyrosine kinases. The physiological and pathophysiological relevance of these effects is not yet clear, but more and more studies indicate that rapid steroid effects on cardiovascular, central nervous and reproductive functions occur in vivo and seemingly transmit physiological and pathophysiological responses.

Future research will have to target the cloning of the first membrane receptor for steroids which should be achieved in near future, and the evaluation of the physiological and clinical relevance of these rapid steroid effects.

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Sex-steroid hormones: cross-talks between receptors and signalling pathways

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The observation that oestradiol through its receptor α stimulates the Src/Shc/Ras/ Erk pathway in MCF-7 and Cos cells disclosed a new aspect of steroid action shared by growth factors and oncogenes1.

We also observed that progestins activate the same pathway in T47D cells2. Surprisingly, antioestrogens prevent progestin activation of the pathway. The cross-talk between PR-B and ERα was also observed in Cos cells. In these cells transiently expressing PR-B, simultaneous expression of ERα is required for a strong stimulation the Src-dependent pathway by progestins. Data indicate that in T47D cells PR-B is preassociated with ER and progestin treatment induces association of ER with Src, apparently responsible for the strong progestin activation of the Src/Erk pathway.

A cross-talk between AR and ERβ was recently detected3. In LNCaP cells androgen or oestradiol stimulates the Src/Ras/Erk pathway. Interestingly, oestradiol antagonists block androgen action and androgen antagonists prevent oestradiol stimulation of the pathway. Each of the two hormones triggers AR/ER /Src association. Experiments in Cos cells show that the co-expression of both receptors reinforces Src activation. ERα shows a behaviour similar to that of ERβ in Cos cells. Phosphotyrosine residue of ERα and a proline-rich stretch of AR are responsible for the association with SH2 and SH3 domain of Src, respectively.
It has been recently observed that oestradiol treatment of MCF-7 cells triggers immediate association of ERα with Src and p85, a regulatory subunit of PI3-kinase4. At the same time Src/PI3-kinase/Akt activation is detected indicating hormone activation of a cross-talk between the two principal signalling pathways. This cross-talk triggers cyclin D1 transcription. Also novel is the finding that Src regulates a PI3-kinase-dependent pathway and PI3-kinase regulates Src activity with a reciprocal cross-talk.

In conclusion, our findings show that each of the sex-steroid hormones stimulates the Src/Ras/Erk pathway; cross-talks between PR-B and ERα and between AR and ERβ trigger a strong stimulation of Src and Src-dependent pathway; a reversible cross-talk between Src and PI3-kinase pathway is induced by oestradiol occupancy of ERα. It should be noted that in all the tested cells activation of the signalling pathways by hormones leads to cell proliferation 3, 4, 5. Transcriptional activity of the oestradiol receptor is not required for signalling activation as well as for DNA synthesis in transfected NIH 3T3 cells, suggesting that this activity is required for later events of cell growth.

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Calmodulin is a selective modulator of estrogen receptors

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Estrogen replacement therapy has been shown to protect women from osteoporosis and other effects of ageing[1-3]. However, it also increases the risk of breast and endometrial cancer[4]. Estradiol effects are mediated by estrogen receptor (ER)α and ERβ. Both receptors transactivate genes regulated by estrogen responsive elements[5]. However, only ERα mediates estradiol dependent transcription of genes regulated by AP1 [5] and Sp1 [6] elements, some of which are implicated in cell proliferation[5-7]. On the other hand, it has been suggested that only ERβ mediates JNK inhibition[8], an alternative mode whereby steroids conduct their immunosuppressive, anti-inflammatory, and antineoplasic pharmacological actions[9]. Therefore, finding selective inhibitors of ERα and ERβ is of paramount importance, both to identify the roles of each receptor and in the therapy of its deleterious effects. Here we show that ERα but not ERβ directly interacts with calmodulin. This is demonstrated by a variety of experimental procedures such us co-immunoprecipitation of *in vitro* labelled ERs with calmodulin, or interaction of pure GST-ER hybrid proteins with dansyl calmodulin. The effect of the calmodulin antagonist W7 on the transactivation properties of the two ERs was examined in the context of an estrogen response element and an AP1 element. ERα was dose-dependent inhibited by W7, whilst the calmodulin antagonist activated ERβ Finally, substitution of lysine residues 302 and 303 of ERα for glycines, rendered a mutant ERα unable to interact with calmodulin whose transactivation activity became insensitive to W7.

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Interaction of estrogen and insulin-like growth factor-I signaling in the nervous system

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Insulin like growth factor-I (IGF-I) is a general trophic factor for neurons and glial cells. Effects of IGF-I are mediated by the IGF-I receptor (IGF-IR), a member of the growth factor tyrosine kinase membrane receptor family that signals through the PI3K pathway and the MAPK cascade. IGF-I regulates the survival and differentiation of neurons and glia and promotes neuronal survival and regeneration after different forms of brain injury. There is now evidence that estradiol, in addition to its participation in neuroendocrine regulation and sexual behavior, has also neuroprotective properties. In different cancer cell lines, IGF-I interacts with estrogen to promote cell proliferation. We have assessed whether estrogen and IGF-I interact in the promotion of neuronal survival and neuroprotection in the central nervous system (CNS).

In a first series of studies, primary neuronal cultures were grown in a defined medium deprived of serum and hormones. Using antisense oligonucleotides for IGF-I and estrogen receptor (ER) alpha, the ER antagonist ICI 182,780 as well as pharmacological inhibitors of the signaling pathways of the IGF-IR, we shown that there is an interdependence of ERs and IGF-IR in the promotion of neuronal survival (1,2). We have also assessed the possible interaction of IGF-IR and ERs in neuroprotection in vivo. Systemic administration of the neurotoxic kainic acid to induce degeneration of hippocampal hilar neurons was used in these studies. Both the systemic administration of 17 beta-estradiol, as well as the intracerebroventricular infusion of IGF-I, prevented hilar neuronal loss induced by kainic acid in adult ovariectomized rats (3). To determine the role of IGF-I receptors in the neuroprotective effect of estrogen we used a specific IGF-I receptor antagonist, the peptide JB1. This antagonist was infused for 7 days in the lateral cerebral ventricle to neutralize the local action of IGF-I in the hippocampus. The neuroprotective action of 17 beta-estradiol was abolished under these conditions. This finding indicates that IGF-IR activation is necessary for the neuroprotective effect of estradiol in this experimental model. Furthermore, the neuroprotective effect of IGF-I was blocked by the infusion in the lateral cerebral ventricle of the estrogen receptor antagonist ICI 182,780, indicating that activation of estrogen receptors is also necessary for the effect of IGF-I on hippocampal neurons (3). These findings suggest that a co-activation of both ERs and IGF-IR is necessary for neuroprotection of primary neurons from serum deprivation, as well as for the neuroprotection of hilar hippocampal neurons from kainate toxicity.

To explore the possible mechanisms involved in the interaction of estradiol and IGF-I in neuroprotection, we examined whether there is an interaction of ER and IGF-IR signaling in the CNS. Analysis of the distribution of ERs, alpha and beta, and IGF-IR in the rat brain by confocal microscopy, revealed that most neurons expressing IGF-IR also express ERs. In addition, some astrocytes co-express ER beta and IGF-IR (4). This finding indicates that interactions of the signaling pathways of ERs and IGF-IR are possible at the cellular level in the CNS, both in neurons as well as in glial cells.

To determine whether there is a cross-regulation of ERs and IGF-IR in the CNS, we infused antagonists of these receptors, or IGF-I, in the lateral cerebral ventricle of adult

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ovariectomized rats. Infusion for 7 days of an ER antagonist (ICI 182,780, 10-7M) resulted in the down-regulation of IGF-IR expression in the hippocampus. This suggests that ERs regulate the expression of IGF-IR in the CNS. We also assessed whether IGF-IR regulates the expression of ERs. Infusion for 7 days of IGF-I (10-4 M) in the lateral cerebral ventricle resulted in a up-regulation of ER alpha in the hippocampus. In contrast, the infusion for 7 days of a IGF-IR antagonist (the peptide JB1, 20 micrograms/ml) resulted in the down-regulation of ER alpha levels. These findings suggest that there is a cross-regulation of ERs and IGF-IR in the rat brain.

We also assessed whether IGF-IR signaling is affected by estradiol in the brain. Estradiol activated in vivo the kinases ERK1, ERK2 and Akt (protein kinase B, PKB), mediators of the effects of IGF-IR. Co-precipitation experiments suggest that the activation of IGF-IR signaling by estradiol may be in part mediated by the interaction of ER alpha and IGF-IR and the consequent phosphorylation of the IGF-I receptor. This interaction is induced by estradiol and probably occurs at the membrane level.

Furthermore, a synergistic action of estradiol and IGF-I in the phosphorylation of Akt was observed. Phosphorylation and activation of Akt results in the phosphorylation and inactivation of the pro-apoptotic molecule Bad and the prevention of apoptosis. Therefore, the synergistic activation of Akt by estradiol and IGF-I in the brain may be involved in neuroprotection.

It is known that the activation of Akt also regulates the expression of the anti-apoptotic molecule Bcl-2. In addition, both IGF-I and estrogen induce Bcl-2 expression in the CNS. We assessed, therefore, whether estrogen and IGF-I may interact in the regulation of Bcl-2 in the brain. We tested whether IGF-IR is necessary for the expression of Bcl-2 in the hypothalamus in response to estradiol. Animals receiving estradiol alone had an increased expression of Bcl-2 in the hypothalamus compared to animals receiving vehicles. However, infusion in the lateral cerebral ventricle of the IGF-IR antagonist JB1 blocked the effect of estradiol on Bcl-2. This suggests that IGF-IR activation is necessary for the induction of Bcl-2 by estradiol in the adult CNS. Therefore, Akt and Bcl-2 may represent molecular targets for the interaction of estrogen and IGF-I in the promotion of neuronal survival.

In summary, these findings indicate that estradiol promotes neuroprotection by genomic actions mediated by the activation of ERs as well as by non-genomic interactions of ERs and IGF-IR. The interaction of ERs and IGF-IR may then result in transcriptional regulation mediated by the activation of IGF-IR signaling.

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Cross talk with signaling pathways as a mechanism regulating ER proteolysis

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Phosphorylation of liganded ER leads to its dimerization, binding to coactivators and transcription of target genes. In addition, ligand binding can mediate cross talk with signal transduction pathways that modulate ER-dependent functions through ER phosphorylation. Estradiol also activates a rapid increase in ER proteolysis. This ligand stimulated ER degradation is thought to be a non-genomic action of the activated ER. The mechanisms leading to proteasome-dependent ER downregulation and identification of proteins involved in this process were investigated.

Within 6 hours of estradiol stimulation of quiescent MCF-7 cells, the ER half-life (t1/2) fell from >8 hrs in G0 cells to 2.5 hrs in mid-G1. Inhibition of the proteasome with N-Acetyl-Leu-Leu-Norleucinal (LLnL) prevented the reduction of ER protein. Inhibition of MAPK kinase with UO126 before estradiol addition further reduced ER t1/2, suggesting that the MAPK pathway inhibits ER proteolysis. Treatment with the Src inhibitor, PP1, caused ER protein accumulation. The role of other signaling pathways to modulate estradiol-dependent ER phosphorylation and degradation is under investigation. Western analysis of ER transfected MCF-7 cells showed predominance of hypo-phosphorylated ER in estradiol-depleted arrested cells. Ligand binding stimulated a shift of the ER to a slightly higher molecular weight. Substrate interaction with the ubiquitin ligase is often triggered by substrate phosphorylation. We have identified a phosphorylation site on the ER whose mutation prevents ligand dependent ER-ubiquitination and degradation. To identify ER associated proteins critical for proteasome mediated ER downregulation, MCF-7 were metabolically labeled. Cells were arrested and then stimulated with estradiol in the presence or absence of LLnL. Two novel ER-bound proteins were detected exclusively in LLnL treated cells. Our results suggest that ER stability may be regulated by phosphorylation via cross talk with signaling pathways. Efforts to identify ER associated proteins that regulate ER proteolysis will be discussed.

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Session 5: Steroid receptor transgenics and knock-outs: lessons from the modellers
Chairs: Günther Schütz and Malcolm G. Parker

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ER receptor knock-outs and reproductive phenotypes

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Targeted disruption of the estrogen receptors in mice has led to several novel discoveries in both males and females. The focus here will be some general phenotypes found in the female mice, but a greater emphasis will be placed on the male. The original ERα knockout (αERKO) has now been shown to express a splicing variant that has a very small amount of estrogenic activity, at least in uterine tissue. A more recent knockout (ERαKO) does not show residual activity; therefore, the two models provide an interesting comparison for studies of reproductive tract biology and estrogen responsiveness. Targeted gene disruption of ERα in both female models resulted in infertility, polycystic ovaries, inhibition of ovulation and uterine hypoplasia. Disruption of ERβ induced subfertility to infertility, inefficient ovarian response and rare CL formation. The double knockout females were also infertile, but showed unexpected ovarian and uterine phenotypes, with the formation of ovarian Sertoli cells and excessive uterine and vaginal hypoplasia. Thus, both ERα and ERβ have important regulatory roles in maintaining fertility in the female reproductive system. In the male, ERβ knockout has no effect fertility, but AR expression is increased in prostate and in the βERKO the prostate epithelium shows hyperplasia. αERKO, ERαKO and the double knockout males are infertile primarily due to inhibition of sexual behavior, but also long-term testicular atrophy.

At puberty there is dilation of seminiferous tubules, rete testis and efferent ductules, indicating abnormal fluid physiology. Normal mice with transplanted αERKO germ cells are fertile; thus, infertility is also due to fluid accumulation and somatic cell dysfunction. The primary αERKO lesion is in the efferent ductules, between the testis and epididymis, where fluid reabsorption is inhibited, causing fluid to accumulate, due to inhibited Na+ transport. In αERKO efferent ductules, epithelial height is decreased with a loss of microvilli and the endocytotic apparatus. However, dysmorphogenesis was not due to the excessive dilation of the lumen, as the ductules in the Nhe3knockout mouse showed greater dilation but normal epithelial morphology. In αERKO efferent ductules, mRNA for Nhe3 is decreased but CFTR is increased. Effects on Aquaporins and Carbonic anhydrase proteins appear to be secondary effects due to the loss of microvilli.

It is concluded that ERα disruption in the male causes infertility by diluting sperm counts and inhibiting normal maturation in the epididymis, a mechanism that could be targeted for developing a new contraceptive for the male.

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Role of cofactors for nuclear receptors in reproduction

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The ability of nuclear receptors to function as ligand-dependent transcription factors depends on the recruitment of cofactors that are involved in chromatin remodelling and the recruitment of the transcription machinery. The repertoire of cofactors required to mediate the action of nuclear receptors is likely to depend on the target gene and cell type. We have focussed on three families of cofactors, namely the p160 family of coactivators, the TRAP220 component of the TRAP coactivator complex and the NRIP1 corepressor. In my presentation I will describe what we have learned about the role of NRIP1 and TRAP220 by analysing the phenotype of mice devoid of these genes.

We have found that NRIP1 is essential for female fertility. Mice devoid of the gene fail to ovulate and yet ovarian follicles undergo luteinisation in response to the LH surge, a process resembling the so-called "luteinised unruptured follicle syndrome " observed in women. The primary site of NRIP1 action in ovulation is in the ovary itself as judged by embryo and ovarian transfer experiments. Its expression increases in granulosa cells as follicles mature but is negligible in luteinised cells. A potential target of NRIP1 may be ERβ whose activity seems to decline following the LH surge. Interestingly mice devoid of the progesterone receptor and COX2 also fail to ovulate and have a phenotype similar to that of NRIP1 null mice. However, since their expression in NRIP1 null and wild-type mice are similar we conclude that the action of NRIP1 is not mediated by either of these proteins and may involve a distinct pathway. Expression profiling has identified a number of genes, including the kallikrein family of proteases that are aberrantly expressed, and so we are investigating their function in detail.

We have confirmed, as reported by others, that TRAP220 is essential for embryonic development at E 9.5 with defects in the placenta and a number of embryonic tissues including the heart. By using tetraploid aggregation assays we have found that wild-type tetraploid blastomeres, which give rise only to placental tissue, partially rescuing TRAP220 null embryos which survive until E 14.5. Thus we conclude that TRAP220 expression is essential for placental function following implantation and in embryonic development after E 14.5

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Genetic dissection of glucocorticoid receptor function in mice

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Most of the effects of glucocorticoids are thought to be mediated by the glucocorticoid receptor (GR) via activation and repression of gene activity. Activation requires binding of a receptor dimer while repression is mediated in many cases by protein-protein interaction of GR with other transcription factors. To analyse glucocorticoid receptor function in vivo several mutations were generated in the mouse. Mice with a disrupted GR gene (null mutation) die shortly after birth due to respiratory failure. To separate activating from repressing functions of the GR a point mutation in the D-loop of the receptor, which is required for receptor dimerization, was generated. Mice carrying this point mutation (GR^{dim}) survive and allowed to distinguish between GR functions dependent on DNA binding and those mediated by protein-protein interaction. Using GR^{dim} mice we were able to demonstrate in primary thymocytes and macrophages that glucocorticoid-dependent repression of cytokine-induction is still functional in the absence of DNA-binding by GR.

Since mice with a disrupted GR gene die after birth, cell-specific mutations have been generated with the Cre-loxP system. The GR gene was inactivated in parenchymal cells of the liver, in thymocytes, monocytes/macrophages, skin and brain, respectively. Unexpectedly, the absence of GR in hepatocytes leads to a dramatic reduction of body size. We could show that growth hormone (GH) signalling mediated by STAT5 in hepatocytes is impaired in these mice leading to lower synthesis of IGF-I and other STAT-dependent mRNAs. Interestingly, GR^{dim} mice show wild-type levels of these mRNAs and have a normal body size. These findings strongly suggest a model in which GR functions as a co-activator for STAT5 transcription upon GH stimulation and reveal an essential role of GC signalling through hepatic GR in the control of body growth. Finally, mice overexpressing GR as a consequence of an increased gene dosage demonstrate that tight regulation of GR expression is crucial for proper control of neuroendocrine and immunological processes.

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Estrogens, estrogen receptors and vasculoprotection

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The incidence of cardiovascular disease, the leading cause of mortality in western societies, is higher in men than in premenopausal women but increases in postmenopausal women. A number of clinical and animal studies strongly suggests a direct effect on the arterial wall.

Endothelium appears to be an important target for estradiol, because this hormone potentiates endothelial NO production, thus promoting the beneficial effects of NO as vasorelaxation and inhibition of platelet aggregation. Estradiol accelerates endothelial regrowth, thus favoring vascular healing, and prevents apoptosis of endothelial cells. Two isoforms of estrogen receptors (α, and ß) have been identified in the cells of the arterial wall. Dupont, Krust et al. (Development 2000, 127 : 4477) have generated mice deficient in ERα due to the deletion of exon 2 (ERα-Δ2 KO). So far, all the vascular effects appear to be mediated by ERα : acceleration of reendothelialization (Circulation 2001, 103 : 423) as well as increase in basal endothelial NO production. Lubahn et al. (PNAS 1993, 90 : 11162) had previously reported the gene inactivation of ERα, consisting in the introduction of a Neo insert in exon 1 (ERαNeo1 KO). In these mice, two ERα mRNA splice variants were detected in uterus and aorta from ERαNeo1 KO mice, one encoding a chimeric ERα55 partially deleted from the A/B domain, and the other an isoform deleted from the A/B domain (ERα46). The clear identification of these two N-terminal truncated isoforms probably explains the persistence of the E2 effects in ERαNeo1 KO mice, which appears as a valuable model to approach the specific roles of the ERα66 compared to ERα46 shown to be physiologically generated in vivo.

Estrogens prevent fatty streak deposit in all animal models of atherosclerosis. In the model of apolipoprotein E-deficient mice, estradiol prevents fatty streak deposit through a mechanism which is clearly independent of NO. The immuno-inflammatory system appears to play a key role in the development of fatty streak deposit as well as in atherosclerotic plaque rupture. Mice deficient in either in monocyte-macrophage or in lymphocytes are partially protected against fatty streak deposit. Interestingly, the atheroprotective effect of estradiol is absent in mice deficient in T and B lymphocytes (Endocrinology 2000, 141 : 462). Thus, the inflammatory-immune system appears to be also a major target of estrogens. However, the effects of estrogens on the immuno-inflammatory system appear ambiguous, as in some models, estradiol rather promotes inflammation.

A better understanding of the mechanisms of estrogens on the normal and atheromatous arteries is required and should help to optimize the prevention of cardiovascular disease after menopause.

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POSTERS

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Modulation of maxi Cl⁻ channels by estrogen and triphenyl-ethylene antiestrogens in c1300 neuroblastoma cells and NIH3T3 fibroblast

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Maxi Cl^- channels are expressed in a wide variety of cell types, however the physiological relevance of these channels remains unresolved. Most electrophysiological studies investigating the properties of Maxi Cl^- channels have been carried out using excised patches, as these channels spontaneously open following patch excision. The addition of triphenylethylene antiestrogens (AE) have been shown to activate these channels in the whole cell configuration, thus permitting the study of these channels in their native environment. We investigated Maxi Cl^- channels in C1300 and NIH3T3 cells and their modulation by AE/oestrogens (E_2) and their intracellular regulation.

Tamoxifen and toremifene (5μM) activated Maxi Cl^- currents as did the non-permeable derivative of tamoxifen, ethyl-bromide-tamoxifen, only when added extracellularly. Pretreatment of cells with E_2 or cAMP prevented the activation of Maxi Cl^- currents by AE. The effect of E_2 and cAMP was abolished in the presence of the serine/threonine (S/T) protein kinase inhibitor staurosporine. Removal of GTP and ATP or addition of 1nM okadaic acid (OA) in the pipette solution also prevented channel activation by AE. These events are consistent with the participation of a S/T phosphatase type-2A (PP2A). To investigate the possibility that modulation of Maxi Cl^- channels are mediated by a de-phosphorylation step, we measured phosphatase activity in cells exposed to AE. Okadaic acid sensitive PP2A-like activity was detected in cells although the PP2A-like activity did not change following treatment with E_2 or AE. Molecular identification of the Maxi-Cl and the presence of estrogen receptors (ERs) was evaluated by immunological detection.

We hypothesize that Maxi Cl^- channels are kept closed by phosphorylation of the channel protein or a regulator associated with the channel. In the presence of AE, the channel is activated by de-phosphorylation through the action of a PP2A-like phosphatase.

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An interaction between androgen receptor (AR) and EGF receptor (EGF-R) disrupts EGF-R-α6β4 interaction and signaling promoting invasion in response to EGF in androgen-sensitive prostate cancer cell lines

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Emerging evidence indicate that androgen-sensitive prostate cancer cells are characterized by a lower malignant potential. We previously demonstrated that androgen receptor (AR) expression by transfection of an androgen-independent prostate cancer cell line (PC3) decreases Matrigel invasion and laminin adhesion of these cells through negative modulation of α6β4 expression (Bonaccorsi et al, 2000).

Treatment with the synthetic androgen R1881 further reduced adhesion and invasion of PC3-AR cells without however modifying α6β4 expression. We investigated here if the AR, upon activation by androgens, has a direct effect on α6β4-EGF receptor (EGF-R) interaction and intracellular signalling leading to invasion of these cells. Immunoconfocal microscopy demonstrated that in control cells transfected only with the vector (PC3-Neo cells), α6β4 and EGF-R co-localized and redistribute at the plasma membrane in response to EGF. In PC3-AR cells co-localization and redistribution between the two molecules was strongly reduced and completely abolished by pre-treatment with R1881. Co-immunoprecipitation studies confirmed the association between EGF-R and α6β4 in PC3-Neo and PC3-AR cells. Notably, tyrosine phosphorylation of both EGF-R and β4 subunit in response to EGF were reduced in PC3-AR cells. To determine whether disruption of EGF-R-α6β4 interaction and signalling in PC3-AR cells was due to an interaction between the AR and the EGF-R, immunoconfocal and co-immunoprecipitation studies of EGF-R and AR were conducted. These studies showed co-localization at membrane level as well as co-immunoprecipitation of the two proteins, indicating an interaction between EGF-R and AR. PI3K activity, a key signalling pathway for invasion, was decreased both in basal conditions and in response to EGF in PC3-AR cells. Most important, treatment with R1881 further reduced PI3K activation by EGF.

In conclusion, in androgen-sensitive PC3 cells, androgens and AR contribute to confer a less malignant phenotype both by reducing the expression of α6β4 (Bonaccorsi et al, 2000) and by interfering with EGF-R-α6β4 interaction and signalling leading to invasion through a direct interaction between AR and EGF-R.

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Two regions of the progesterone receptor interact with the estrogen receptor and are selectively required for activation of the MAP kinase Erk1/2 by progesterone

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In addition to direct effects on gene transcription, steroid hormones influence cell physiology by crosstalk with various signal transduction pathways. In breast cancer cells estrogens have been shown to activate the Src-Ras-Raf-MEK-Erk signalling pathway through an interactions of the estrogen receptor (ERa) with c-Src. Progesterone also activates this cascade but, although it has the potential to interact with the SH3 domain of c-Src, in T47D cells it activates the Src-Ras-Raf-MEK-Erk signalling pathway indirectly, via an interaction of the progesterone receptor (PRB) with ERa. Using the yeast two-hybrid assay we demonstrate here that two distinct regions on the N-terminal half of PRB mediate this interaction. One region is located between amino acids 165 and 345 and overlaps partly with a previously described inhibitory domain. The other region extends from amino acid 456 to 546 and coincides with the activation function I. The interaction between PRB and ERa is direct, since it can be observed in GST pull-down experiments with recombinant proteins. Both regions of PRB interact with the ligand binding domain of ERa, and each region individually is sufficient for the interaction. In COS-7, the interaction between both receptors can be demonstrated by co-immunoprecipitation and is abolished by deletion of any one of the two in vitro interacting regions of PRB. Both deletion mutants of PRB as well as the double mutant are as effective as the wild type PRB in the activation of a cotransfected MMTV-luc reporter gene in response to hormone. However, whereas in cells cotransfected with ERa, PRB activates the MAP kinase Erk, neither the individual PRB deletion mutants, nor the double mutant exhibited any activity in this assay. Thus, the two regions of PRB mapped in this study appear to be involved in progesterone activation of the Src-Ras-Raf-MEK-Erk signalling pathway via ERa.

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Activation of the phosphatidyl-inositol-3-kinase/AKT signalling pathway by retinoic acid is required for neural differentiation of SH-SY5Y human neuroblastoma cells

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Differentiation of mammalian cells is a complex process that is regulated by the interplay of intrinsic gene expression programmes and external signals provided by hormones and growth factors. Nuclear Hormone Receptors (NHR) and their ligands play a key role in this process. Retinoic Acid (RA) leads to growth arrest and promotes differentiation of human neuroblastoma cell line SH-SY5Y. RA treatments result in a co-ordinated downregulation of the expression of the ID-HLH genes, a subfamily of HLH transcription factors. ID-HLH factors act as inhibitors of cell differentiation by interfering the actions of the differentiation-promoting bHLH factors. Transcriptional repression of ID gene expression by RA involves a complex mechanism. Downregulation of the ID genes requires newly synthesized proteins and the activity of the phosphatidyl-inositol-3-kinase (PI3K). RA treatment of neuroblastoma cells leads to activation of the PI3K/AKT signalling pathway, as demonstrated by an in vitro kinase assay and immunodetection of phosphorylated AKT. Inhibition of PI3K by its specific inhibitor LY294002 impairs neural differentiation, as assessed by morphological (inhibition of neurite extension) and molecular (block in the induction of the expression of GAP43) criteria. We propose that this non-genomic activation of the PI3K/AKT signalling pathway by RA provides a coupling between neural differentiation and cell survival, supporting limited survival for newly differentiated cells until it could be provided by neurotrophic factors. The mechanism through which RA activates the PI3K/AKT signalling pathway remains to be established, and we are trying to demonstrate direct interactions between RAR and p85, the regulatory subunit of PI3K.

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Characterization of non genomic effects of estrogens in HeLa and MCF7 cells

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Estrogens are steroids with multiple beneficial and harmful biological effects which are essentially elicited through two signaling pathways. The first one is classically referred to as the genomic pathway and involves nuclear estrogen receptors ER∀,∃ and estrogen receptor-related (ERR) polypeptides. The second estrogen signaling pathway is characterized by the estrogen-dependent activation of cytosolic signaling pathways such as PKB/Akt and the MAPK signaling modules, which are critical in eliciting favorable effects of estrogens on blood vessels for example. The nature and characteristics of the molecular relays for such biological responses is yet debated. Using defined cellular backgrounds and a panel of natural and synthetic estrogens and anti-estrogens, we have undertook a comparative study between genomic and non genomic effects of these compounds. Our results indicates that pharmacological properties of estrogens are distinct when considering both types of responses, and preliminary data concerning the molecular characterization of estrogen effectors will be presented.

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Acute modulation of acetylcholine-induced calcium signals by estradiol in GT1-7 Cells: Evidence for membrane binding sites

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Estrogen regulation of the female pituitary-gonadal axis involves the rapid inhibition (< 30 min) of luteinizing hormone-releasing hormone (LHRH) secretion from hypothalamic neurons. Given its fast time course this effect is unlikely due to the activation of nuclear estrogen receptors. Rather, it is possible that interactions with potential binding sites at the plasma membrane may result in short-term estrogen effects on LHRH neurons. We have studied the acute effects of 17β-estradiol (E_2) on the elevations of intracellular calcium concentration ($[Ca^{2+}]_i$) induced by acetylcholine (ACh) in GT1-7 cells. Exposure to ACh (100 μM) induced transient increases of $[Ca^{2+}]_i$, while pretreatment with E_2 (10 nM) for 3 min reduced this response by half. Since the latency of this effect suggested a membrane-mediated mechanism, we investigated the existence of membrane binding sites for E_2 in GT1-7 cells. This was evidenced by means of assays using estradiol-peroxidase (E-HRP) and estradiol-BSA-fluorescein isothiocyanate (E-BSA-FITC) conjugates. Binding of these compounds to the plasma membrane was blocked by preincubating cells with E_2. We conclude that GT1-7 cells do respond to ACh with an increase in $[Ca^{2+}]_i$. This ACh-induced Ca^{2+} signal is acutely modulated by physiological E_2 concentrations in a manner which is compatible with the existence of an estrogen especific membrane binding site. These results are indicative of an acute, membrane-mediated mechanism by which estrogen may modulate the response of LHRH neurons to a cholinergic presynaptic input.

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Estrogen receptor β involvement in cell adhesion signalling and gap junctional communication in differentiating mammary gland

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Despite the clear role for estrogen in proliferation of the mammary epithelium, the role of estrogen receptors in this process remains unclear (1, 2). The phenotype of the mammary glands of adult virgin BERKO mice reflects the action of ERa in the absence ERβ, i.e. a significant and distinct role for ERβ in the response of the mouse mammary gland to progesterone and as a pro-apoptotic signal molecule (3, 4).

The mammary gland is a unique organ in that full differentiation is not attained by adulthood. During pregnancy, induction of functional differentiation takes place under the influence of high estrogen and progesterone levels and increasing prolactin and placental lactogen levels, as a result of which the mammary gland becomes secretory (5). To get deeper insight into the role of ERbeta in mammary gland biology, we started to look at the differentated organ during lactation using BERKO mice. During the process of mammary gland remodelling, no proper zona occludens/ adherens and nexus communication is formed in lactating BERKO mice. Classical cadherins such as E-, N-, and P-cadherin, play critical roles in tissue morphogenesis (6). Cadherin E and P expression, the major adhesion receptors of the zonula adhaerens junctions of epithelia, is changed in lactating BERKO breast. Cadherins dynamically regulate the free β-catenin pool active in signalling by association of its cytoplasmic tail with catenins (7). Catenins may be translocated into the mucleus and influence transcriptional activity of genes like c-myc, cyclin-D1, matrilysin, c-jun and c-fra, which might explain the observed expression of the proliferation marker Ki-67 in lac BERKO but not in wild type breast.Cadherin-cadherin interactions trigger furthermore cell type-specific intracellular signal cascades resulting in gap junctional communication/ plaque formation. The wild type lac mammary gland shows a regular pattern of tight junctions (zona occludens) and gap junctions (nexus). Tight junctions between alveolar cells allow the formation and maintainance of a milk that is compositionally distinct from blood (8). Gap junctions render the alveolar units electrically coupled and have a dual communication and adhesion function, allow cell-to-cell traffic of second messengers, e.g. IP3, Ca^{2+} in non-excitable tissues. The formation of tight and gap junctions is greatly diminished in lac BERKO glands. Generally, integrin levels,comprising cell-matrix adhesion, are reduced in many breast cancers (9). The expression of integrin a2 expression is diminished, more diffuse and disorganized in lac BERKO mammary glands.

The present findings provide the first direct evidence for a causal role of ERβ in the maintainance of normal tissue morphology, epithelial organization and adhesion signalling, and in the control of tumor suppressor and oncogenes in breast tissue.

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Sexual hormones receptors in glial cells after brain injury

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Neuronal degeneration is accompanied by the activation of glial cells. Activated astroglia express a large number of molecules implicated in the response of brain tissue to injury. Reactive astrocytes are recognized by the expression of Vimentin, increased cellular volume and an increased expression of glial fibrillary acidic protein (GFAP). Although under normal circumstances alpha estrogen receptors (ERalpha) are almost exclusively expressed in neurons, estrogen is known to be able to regulate the proliferation and the morphology of reactive astroglia. To test if estrogen could affect directly the function of reactive astrocytes, we have evaluated the expresión of (ERalpha) in glial cells after a stab wound brain injury using optical and confocal microscopy.

After the injury, we observed the co-expression of ERalpha with specific markers of reactive astrocytes (GFAP and Vimentin). This co-expresion started three days after the injury, reached its maximal level at day 7 and remained until day 28. ERalpha immunoreactivity appeared in the glial cell nucleus and also in the cytoplasm and processes of reactive astrocytes.

These results point to a direct action of estrogen on reactive astroglia and suggest a possible non-genomic mechanism for these effects.

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17β-estradiol prevents β-Amyloid1-40-induced toxicity in murine septal cells: Evidence that intracellular estrogen receptors mediate this neuroprotective effect

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Alzheimer's disease is a neurological disorder characterized by a progressive regression of memory and cognitive functions, together with the dysfunction of cholinergic neurons. One of the early events of this neurodegeneration is the presence of neuritic plaques predominantly formed by fragments of β-amyloid peptide (βA) that, when accumulated, seem to be responsible for a progressive neuronal degeneration. At the cellular level, this phenomenon constitutes a useful pharmacological tool for the development of in vitro neurodegenerative models, as well as for the characterization of potential protective mechanisms. On the other hand, an increasing number of experimental evidences demonstrate that estrogen is capable of certain neuroprotective actions that may be exerted, at least in part, through genomic mechanisms related to nuclear estrogen receptors (ERs). In the aim of contributing to the development of new therapeutic strategies, we have studied the effect of estrogen and the potential involvement of ERs in this phenomenon, using a septal cell line (SN56) known to produce LHRH, acetylcholine and nitric oxide, and to express functional ERs1-3.

To assess ER functional activity, SN56 cells were transfected with an ERE2-tk-Luc construct, consisting of two estrogen response elements controlling luciferase expression in a pGL2 plasmid, and then exposed to increasing concentrations of 17β-estradiol (E2) for 24 h. Luciferase expression in transiently transfected cells increased with E2 concentration in a dose-related manner, a response that was blocked by addition of ICI182,780. Then, to evaluate estrogen neuroprotective activity in these cells, they were incubated for 24 h with fragment 1-40 of beta-amyloid peptide (βA1-40, 50 μM), alone or together with different concentrations of E2 (10 pM-100 nM). Exposure to βA1-40 provoked massive cell death (75%) that was prevented by physiological estrogen concentrations in a dose-dependent manner (7% mortality at 100 nM E2). The neuroprotective estrogen effect was blocked by the ER antagonist ICI182,780, and was not observed when using identical concentrations of the non-physiological isomer 17α-estradiol. In addition, we observed up-regulation of ERα during cell injury, as demonstrated by RT-PCR and Western blotting. Taken together, these results suggest that the neuroprotective action of E2 against βA in murine cholinergic neurons may be mediated by its interaction with a classical nuclear estrogen receptor

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Cross-talks between heregulin and progesterone receptor signaling pathways in mouse mammary adenocarcinomas

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Activation of the progesterone receptor (PR) by heregulin was studied in an experimental model of hormonal carcinogenesis, in which the synthetic progestin medroxyprogesterone acetate (MPA) induced mammary adenocarcinomas in female Balb/c mice. We have previously demonstrated that HRG stimulates growth of primary cultures of the progestin-dependent C4HD epithelial cells and potentiates MPA mitogenic effects (Oncogene 18: 6370-6379,1999). In the present study, we found that HRG(20ng/ml) treatment of C4HD cells induced a decrease of protein level of PR A and B isoforms and down-regulation of progesterone-binding sites. HRG also promoted a significant increase in the percentage of PR localized in the nucleus. DNA mobility shift assay showed that HRG was able to induce PR binding to a progesterone response element (PRE). When C4HD cells were transiently transfected with a plasmid containing a PRE upstream of a chloranfenicol acetyltransferase (CAT) gene, HRG induce a significant increase in CAT activity. In order to assess molecular mechanisms underlying PR transactivation by HRG, we pretreated C4HD cells with the selective MEK 1 inhibitor PD98059 (10uM). We found that PD 98059 blocked HRG capacity to inducePR binding to a PRE and CAT activity.We next studied the ability of MAPK to phosphorylate PR. In vitro phosphorylation assays showed that active MAPKs isolated from HRG treated cells were able to induce PR phosphorylation which was inhibited after PD 98059 treatment. As control for PR activation, in all experiments described, C4HD cells were treated with MPA demonstrating that HRG effects on PR activation were comparable to those exerted by MPA. These results provide the first evidence that in the absence of progestins, the cognate ligands, HRG activates PR by a mechanism that requires MAPK activation.

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Androgen control of gene expression in lacrimal glands of normal and autoimmune mice

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Androgens regulate the structure and function of the lacrimal gland and are responsible many of the sex-related differences in this tissue. Androgens also dramatically suppress the inflammation in lacrimal glands of the MRL/lpr mouse model of Sjögren's syndrome. We hypothesize that these androgen effects are mediated primarily through nuclear androgen receptors and involve the control of specific target genes. The purpose of this investigation was to begin to test this hypothesis.

Methods. Lacrimal glands were obtained from intact or orchiectomized BALB/c and MRL/lpr mice, that were untreated or administered vehicle or testosterone for 2 weeks, as well as from *Tfm* mice with dysfunctional androgen receptors and their Tabby controls. Tissues were processed for the identification of gene expression patterns by using a modified differential display of 3'end cDNA restriction fragments and by utilizing Incyte gene chips (> 8,000 genes).

Results. Analysis of differential display gels with BALB/c and *Tfm*/Tabby samples led to the identification of ~300 lacrimal gland genes that appear to be up- or down-regulated by androgens. Use of gene chip technology permitted identification of many additional androgen-controlled genes in lacrimal tissues of normal and autoimmune mice, and number of these androgen-gene interactions seemed to be mediated through the androgen receptor.

Conclusions. Our results show that androgens regulate the expression of numerous genes in the mouse lacrimal gland.

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The naturally occurring ERΔE7 variant suppresses estrogen-dependent transcriptional activation by both wild type ERα and ERβ

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More than 50% of breast cancer are ER and its growth is initially dependent on estrogens and inhibited by antiestrogens, being the presence of functional estrogen receptors essential for their response to both estrogens and antiestrogens. The existence of different variants generated by alternative splicing of ERα1 and ERβ2that function as dominant negative has been interpreted as a physiological protective mechanism of regulating the E2-dependent growth of responsive tissues and, alternatively, as a deletereus mechanism that render the ER+ cancer cells resistant to antiestrogen therapy3-5.

With regard to the exon 7-skipped variant, contrasting results depending on the transfection assay system used have been obtained. Thus, ERΔE7 variant, which was reported to repress 60% of the action of equimolar wild-type ER in yeast6 is apparently ineffective as a dominant negative inhibitor in mammalian cells5. Our work focuses on the functional characterization of the exon 7-skipped variant isolated from MCF-7 cells as the most abundant splicing form of ERα expressed in this estrogen receptor-positive mammary carcinoma cell line and in breast tumors.

We have generated the recombinant plasmids pGTK-LBD and pGTK-LBDΔE7,containing the HBD/AF2 of wt hERα (residues 280-595) and the corresponding sequence of the Δ7 squiped receptor. (residues 280-457 plus 10 non ERα residues). as well as the expression plasmids pcDNA-ERα and pcDNA-ERΔE7 that contain the full-length cDNAs of wild type ERα and ERΔE7 variant and made the following observations:

1).- The ERΔE7 variant exhibits at least a 100-fold reduction in its ability to bind estrogen (as expected since the exon 7 deletion eliminates a significant portion of the LBD).

2).-The ERΔE7 variant forms heterodimers with both wild type ERα and ERβ. in a ligand-independent manner as demonstrated by different procedures.

3).- The ERΔE7 variant inhibits the E2-dependent transcriptional activation of genes regulated by ERE pS2 promoter sequences mediated by both wild type ERα and ERβ. In both experiments we observed that increasing amounts of ERΔE7 resulted in a progressive inhibition of the E2-dependent induction of luciferase activity by both ERα and ERβ..

4).- To investigate whether inhibition of transcription by ERΔE7 is exerted at level of DNA binding, we have compared the ability of ERΔE7 and wild-type receptors to bind to the ERE *in vitro*. The results indicate that the ERΔE7 variant blocks the binding of wild type ERα and ERβ to their responsive element as determined in transfected HeLa cells.

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Estrogen and insulin-like growth factor I (IGF-1) signalling pathways interact in the central nervous system

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Estrogen has protective and trophic effects for a variety of cell types in the Central Nervous System (CNS), but the mechanisms for this neuroprotection are largely unknown. The Insulin-like Growth Factor-I also acts as a trophic factor for neurons and glial cells and acts by binding to a Tyrosine Kinase Receptor (RTK). Estrogen and IGF-I interact in the control of cell proliferation in peripherical organs and there is a wide co-expression of the receptors for both hormones in the CNS.

In this work, we have studied the effect of estrogen on the IGF-I receptor signalling pathways in the CNS in vivo. An acute estrogen treatment in ovariectomized rats increase the activation of Akt/Protein Kinase B in different brain areas (hippocampus, hypothalamus and hypophysis) as measured by Western-Blotting with specific antibodies. This treatment also increases the level of tyrosine phosphorilation of the IGF-I receptor and induces the association between this receptor and the estrogen receptor alpha.

This results suggest a new mechanism for estrogen neuroprotection and demonstrate a interaction between estrogen and the signalling pathways used by RTK.

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Effect of 17β-estradiol on vascular toxicity induced by β-amyloid fibrils

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Amyloid β-peptide (Aβ) fibril deposition on cerebral vessels produces cerebral amyloid angiopathy which appears in the majority of Alzheimer's disease patients. An early onset of a cerebral amyloid angiopathy variant called hereditary cerebral haemorrhage with amyloidosis of the Dutch type (HCHWA-D) is caused by a point mutation in Aβ yielding $A\beta_{Glu22\rightarrow Gln}$. The present study addresses the effect of amyloid fibrils from both wild-type and mutated Aβ on vascular cells as well as the putative protective role of 17β-estradiol on amyloid angiopathy. For this purpose, we have studied the cytotoxicity induced by $A\beta_{1\text{-}40\ Glu22\rightarrow Gln}$ and $A\beta_{1\text{-}40}$ wild-type fibrils on human venule endothelial cells (HUVEC) and vascular smooth muscle cells (A7r5). We observed that $A\beta_{Glu22\rightarrow Gln}$ fibrils are more toxic for vascular cells than the wild-type fibrils. We have also evaluated the cytotoxicity of Aβ fibrils bound with acetylcholinesterase (AChE), a common component of amyloid deposits. $A\beta_{1\text{-}40}$ wild-type -AChE fibrillar complexes, similar to neuronal cells, resulted in an increased toxicity on vascular cells. In the search for approaches that might ameliorate the vascular toxicity of Aβ fibrils, we tested if 17β-estradiol was able to reduce the vascular damage induced by Aβ. 17β-estradiol has been reported to inhibit the toxicity of Aβ and $A\beta_{1\text{-}40}$-AChE fibrils on neuronal cells. However, our data indicate that 17β-estradiol failed to inhibit the cytotoxicity induced by Aβ fibrils on vascular cells.

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Apoptosis and steroid hormones: Glucocorticoid-mediated bcl-X expression in mouse thymocytes

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Apoptotic response to steroid hormones has been studied in different tissues, however little is known about the genes and the mechanisms which are involved in this process. Bcl-2 related genes have been demonstrated to control apoptosis in several cell types (1). Bcl-X is one of the members of this family and its expression generates different isoforms with oposite functions (2). The steroid hormones dexamethasone and progesterone have been shown to induce bcl-X expression, and to increase the ratio bcl-XL (antiapoptotic)/bcl-XS (proapoptotic) in those tissues where they inhibit apoptosis (3,4). Mouse bcl-X gene contains at least five different promoters which are activated in a tissue-specific pattern of promoter usage. Promoter selection would influences the outcome of the splice process (5). We studied the molecular mechanism involved in the glucocorticoid-mediated bcl-x expression in murine thymocytes. RT-PCR were performed to analyze the relative levels of bcl-X isoforms. The results have shown that dexamethasone provokes a decrease in the ratio bcl-XL/bcl-XS favoring the expression of the proapoptotic isoform. This effect has been observed only in those mRNA generated upon the induction of some distal promoter located upstream promoter 2. Transient transfection assays of expression vectors containing the reporter gene luciferase under the control of each bcl-X promoter have shown that promoter 4 (P4) responds specifically to the glucocorticoid, suggesting that P4 plays a key role in the hormone-mediated expression of bcl-XS. Since no consensus hormone regulatory element motifs are found in the neighborhood of this promoter, our results suggest that steroid receptors might control bcl-X expression by interacting with other transcription factors.

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Altered skin development and impaired proliferative and inflammatory responses in transgenic mice overexpressing the glucocorticoid receptor

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We addressed to study the role of the glucocorticoid receptor (GR) in several ectodermal derivatives by generating transgenic mice that overexpress this protein, which mediates the biological effects of the glucocorticoids, under the control of the keratin K5 promoter. K5-GR mice allowed us to study developmental and pathophysiological aspects of GR function in these tissues. K5-GR transgenic mice were generated by standard procedures. At 18.5 d.p.c., all transgenic mice showed reduced embryonic growth, abnormally smooth and thin skin, and eyelid colobomas with defects of the upper/lower eyelid. Detachment of the retina was apparent in transgenic head sections, neither the conjunctival sac nor the anterior chamber of the eye existed, the hyaloid cavity was completely distorted and the lens displayed evident symptons of necrosis. Adult transgenic mice displayed severe anomalies ranging from remarkably corneal opacity to the absence of ocular structures. In the most affected individuals, skin was absent at the cranial and umbilical regions, and the vibrissae and eyebrows appear scarce, short and curly. We have observed multiple anomalies in the oral cavity of these transgenic mice and cleft palate in the most severely affected mice that paralleled the effects of an early overexposure of fetuses to glucocorticoids. All together, K5-GR mice exhibited a phenotype which strikingly resembles the clinical findings in patients with ectodermal dysplasia (ED), which includes aplasia cutis congenita (ACC). The present work provide, to our knowledge, the first evidences supporting that unbalanced glucocorticoid function lies onto the molecular basis of the relevant group of human disorders collectively termed Ectodermal Dysplasia.

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A nonclassical estrogen membrane receptor triggers rapid differential actions in the endocrine pancreas

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Glucose homeostasis in blood is mainly maintained by insulin released from b-cells and glucagon released from a-cells, both integrated within the pancreatic islet of Langerhans. The secretory processes in both types of cells are triggered by a rise in intracellular calcium concentration ([Ca2+]i). In this study, rapid effects of the natural hormone estradiol on [Ca2+]i were studied in both types of cells within intact islets using laser scanning confocal microscopy. Alpha and beta cells showed opposite [Ca2+]i responses when stimulated with physiological concentrations of 17b-estradiol. While the estrogen produced an increase in the frequency of glucose-induced [Ca2+]i oscillations in insulin-releasing b-cells, it prevented the low glucose-induced [Ca2+]i oscillations in glucagon-releasing a-cells. Evidence indicated that these were membrane actions mediated by a nonclassical estrogen receptor. Both effects were rapid in onset and were reproduced by 17b-estradiol linked to horseradish peroxidase (E-HRP), a cell-impermeable molecule. Furthermore, these actions were not blocked by the specific estrogen receptor blocker ICI 182,780. Competition studies performed with E-HRP binding in a-cells supported the idea that the membrane receptor involved is neither ERa nor ERb. Additionally, the binding site was shared by the neurotransmitters epinephrine, norepinephrine and dopamine and had the same pharmacological profile as the receptor previously described for b-cells. Therefore, rapid estrogen actions in islet cells are initiated by a nonclassical estrogen membrane receptor (ncmER).

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Tissue specific regulation of Ca^{2+} channels by sex hormones. Genomic regulation of a calcium channel protease?

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We studied protein expression levels of alpha1C L-type Ca^{2+} channels in uterus, brain and heart, comparing estrus non pregnant (NP) and late pregnant (day 21, P21) rats. Two forms of alpha1C were detected in uterus, brain and heart, the alpha1C-long of ~240 kDa and the alpha1C-short of ~190 kDa, which is a functional proteolytic product by cytoplasmic proteases. In P21 uterus, alpha1C-long doubled the expression of alpha1C-short; in NP the opposite occurred. However, the total alpha1C protein remained constant. No changes were detected in brain and heart. To determine which sex hormones regulate Ca^{2+} channel expression in a tissue-specific manner, we determined alpha1C protein levels in uterus, brain and heart in ovariectomized rats (OVX) treated with sex hormones. After 4 day treatment (2 injections/day) with sesame oil (control), 0.6 mg progesterone (P4), 10 mg ß-estradiol (E2), and P4+E2, changes in protein levels were analyzed using Western blots. Progesterone treatment of ovariectomized (OVX) rats, yielding high P4/E2 ratios, facilitates expression of alpha1C-long in myometrium. Whereas estrogen treatment, yielding low P4/E2 ratios, favored the alpha1C-short form. Total alpha1C protein remained constant. Neither hormone altered alpha1C expression in brain or heart. Thus, in late-pregnancy, a large P4/E2 ratio is one mechanism that governs alpha1C-long expression in a tissue-specific manner. In conclusion, the total amount of L-type Ca^{2+} channels in myometrium does not change at the end of pregnancy; rather, the ratio of the long/short forms is increased via a progesterone-mediated mechanism. In non-pregnant rats at estrus, estrogen is involved in the predominant expression of the short form. This hormonal control is tissue specific since heart and brain tissues do not undergo significant changes. In myometrium, a higher amount of alpha1C-long form one day before partum would traduce in Ca^{2+} channels with reduced activity assuring that gestation reaches term at a stage when K^+ (MaxiK and Kv4.3) channels are already reduced. It is evident that a tight hormonal control by progesterone and estrogen of membrane ion channels expression is necessary for a successful pregnancy and delivery, and that preventive mechanisms exist to avoid large heart and brain remodeling by increased circulating sex hormones during pregnancy. Progesterone and estrogen regulation of α_{1C} isoforms could be mediated by a genomic regulation of a Ca^{2+} channel protease that would transform the long into the short form of alpha1C. Supported by NIH.

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LIST OF INVITED SPEAKERS

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