386P A COMPUTER SIMULATION OF EXPERIMENTS TO DEMONSTRATE PHARMACOLOGICAL CONTROL OF GUINEA PIG AIRWAYS

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Computer programs which simulate undergraduate pharmacological experiments are now widely available, and most undergraduate pharmacology courses in the UK employ examples of them.

Here we demonstrate a highly interactive computer simulation designed to demonstrate the action of a number of pharmacological agents and procedures on the lung function of the anaesthetised guinea pig and teach the basic pharmacology of the airways to undergraduate students on courses in which pharmacology is a major component. Data from actual experiments has been used throughout and are based on recordings made using a digital electronic pulmonary monitoring system (PMS: Mumed Ltd, London, UK). This system monitors tracheal airflow and transpulmonary pressure from which a number of lung function parameters may be measured e.g. resistance and dynamic compliance. The actions of a number of mediators and antagonists/inhibitors may be investigated in normal and allergic guinea pigs, the latter having been sensitized by previous administration of ovalbumin.

The program was developed using Macromedia Director (version 7) for PCs (minimum specification: Pentium PC, Windows 95/98/NT4, 16 Mb RAM, 10Mb available HD space 16 bit colour graphics).

The 'Introduction' section combines text and colour graphics and covers: aims and objectives, innervation of airway structures and receptor pharmacology of bronchial smooth muscle, the mechanism of allergen sensitization and includes information and a quiz on the agents and drugs used in the program. 'Methods' describes the animal preparation, and the measurement of airway compliance, resistance and blood pressure. The 'Experiments'section allows the student to select, from a menu, to study the effects of various mediators and inhibitory agents in the normal and allergen-sensitized animals.

Simultaneous traces of resistance, dynamic compliance and blood pressure are presented in a form similar to that in the Mumed recording system and each set of data is accompanied by self-assessment questions which demand interpretation of experimental data presented to them, and an understanding of the underlying control mechanisms. These student-centred activities make the program useful for self-directed learning or, in the ideal situation, it would be incorporated into a structured teaching programme and used with a teacher-designed workbook.

It is envisaged that the program could be used in a number of ways: to better prepare students who will perform the practical at a later date; to debrief students after they have performed the practical; as a 'fallback' to provide data for students whose experiments were unsuccessful; as an alternative to the practical, though it should be remembered that different learning objectives may be achieved.

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387P CHEMOKINES AND THEIR DIVERSE BIOLOGICAL PARADIGMS

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Scientific advances continue to identify members of the chemokine supergene families as biologically diverse mediators of important physiologic events. While initial investigations originally defined the biological activity of chemokines as proteins with novel chemotactic activity for specific sub-populations of leukocytes, data now supports a much broader biological role for the chemokines.

The chemotactic activity of chemokines for specific leukocyte sub-populations is, in itself, an important activity, as this response provides a mechanism for the successful delivery of the appropriate leukocyte population from the lumen of the vasculature to a site of inflammation. This biological response provides the means for the accumulation of either granulocytes at foci of acute inflammation, via the activity of CXC chemokines, or the accumulation of mononuclear cells at foci of chronic inflammation, via the activity of CC chemokines.

However, leukocyte chemotaxis may not be the only, or the most important, activity of the chemokine family members. A variety of reports have stressed the key role of chemokines in a variety of physiologic and pathologic situations, which may provide mechanisms for activating cytokine networks, altering the expression of adhesion molecules, increasing cell proliferation, regulating angiogenesis, promoting viral-target cell interactions, haematopoiesis and activating the innate immune system. The importance of chemokines as a contributing player to the immune response is further underscored by recent investigations that have identified viral genes that encode chemokine binding proteins.

Chemokines have also been shown to participate in the progression of chronic inflammation by influencing mononuclear cell chemotaxis, hematopoiesis, angiogenesis, stromal cell proliferation, matrix deposition and lymphocyte polarization. This latter activity is especially important, as specific chemokine ligand/receptor pairs have been identified in type 1 (Th1) versus type 2 (Th2) immune responses. For example, investigations have identified that type 1 responses are promoted by the participation of the following chemokine ligands: MIP-1 alpha, RANTES, IP10, Mig, and ITAC and the chemokine receptors: CCR1, CCR5, CXCR3; while type 2 response are driven in part by the participation of eotaxin, MDC, MCP, TARC, I-309, SDF and the chemokine receptors: CCR2, CCR3, CCR4, CCR8, and CXCR4.

These observations have played an important role in the design of efficacious small molecular weight antagonists to therapeutically target specific chemokine receptors, as these receptors and their ligand pairs are likely to participate in the evolution of chronic immune response.

388P CHEMOKINE RECEPTORS AND DRUG DEVELOPMENT: THE CHALLENGES

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The trafficking of pro-inflammatory leukocytes is regulated by a complex, multi-step process involving cell-cell adhesion, protein interactions between leukocytes and vascular endothelial cells, chemoattractant factors and their receptors on the surface of leukocytes, and cell movement. During the past decade, significant advances have been made in our understanding of this process and the factors that play critical roles in the regulation of the extravascular recruitment of leukocytes. In particular, the discovery and study of the chemokine (for chemoattractant cytokine) superfamily has provided a clearer understanding of some of the mechanisms by which the migration of leukocytes is controlled in pathological settings as well as during normal immune function as well as in pathology.

The activity of chemokines is mediated by cell surface receptors that comprise a subfamily of the G protein-coupled receptor (GPCR) superfamily. Following engagement of their receptors, which primarily couple to Gαi, chemokines activate several signal transduction pathways that induce a variety of functional responses including cellular chemotaxis, granule release, superoxide production, integrin up-regulation, cellular differentiation, kinase activation, and proliferation.

Validation of the chemokine systems as new therapeutic targets for inflammation has advanced prodigiously during the latter part of the last decade. The development of a plethora of chemokine and chemokine receptor reagents, such as, blocking monoclonal antibodies, modified antagonistic chemokines, and genetically modified mice has allowed correlations to be made between chemokines and the induction or maintenance of disease. Many chemokines are also upregulated in various pathologies, including, rheumatoid arthritis (RA), multiple sclerosis, atherosclerosis, asthma, chronic obstructive pulmonary disorder (COPD), and allergic disease, to name a few.

Taken together, this information has provided a very strong rationale for the implementation of chemokine antagonist programs. Although sometimes difficult to ascertain, there are a few examples of chemokine receptor antagonists moving into the clinical realm. However, these receptors have also proven to be relatively difficult to drug, as compared to small molecule binding GPCRs, and so different approaches to antagonist discovery have been undertaken. Several difficulties encountered in chemokine receptor drug discovery will be highlighted, along with an example of our progress in the discovery of a potent antagonist monoclonal antibody to CCR2, and an example of the discovery of potent small molecule antagonists of CCR1.

389P ROLE OF CXCR2 ANTAGONISTS IN INFLAMMATORY DISEASE

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A potential strategy for the therapeutic intervention in a number of inflammatory diseases would be to control the recruitment and activation of inflammatory cells.

For many of these diseases, neutrophils and T-cells and the products they release upon activation appear to be important for the pathophysiology. IL-8 and related CXC (ELR^+) chemokines (GROα and ENA-78) are proinflammatory chemokines that attract and activate immune and inflammatory cells and are present at elevated concentrations in several inflammatory diseases.

The effects of IL-8 on inflammatory cells are mediated through two receptors, CXCR1 and CXCR2, which are members of the superfamily of G protein-coupled, seven transmembrane spanning receptors. We have recently identified a class of non-peptide compounds that are potent and selective antagonists of CXCR2. The process by which leukocytes infiltrate inflammed tissue appears to be mediated, at least in part, by CXCR2.

This presentation will include data in support of the role of IL-8 and CXCR2 in inflammatory disease, as well as, a description of the efficacy of a potent serlective CXCR2 antagonist in animal models. The results indicate that selective CXCR2 antagonists may be useful to define the role of this receptor in inflammatory diseases.

390P CCR5 ANTAGONISTS WITH ANTI-HIV ACTIVITY: IS INHIBITION ON THE CELL SURFACE SUFFICIENT?

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Discovery that interaction between the viral envelope protein gp120 and the chemokine receptor CCR5 was necessary for fusion and entry of M-tropic HIV strains not only enhanced understanding of the infection process but also provided a novel molecular target for therapeutic intervention. However, during efforts to develop CCR5 antagonists it became evident that simple inhibition of the CCR5-gp120 interaction was not sufficient to block infection as many nanomolar and sub-nanomolar inhibitors had little or no antiviral activity, while other similar inhibitors were potent antivirals.

The discordance appears to arise from the nature of the CCR5-viral interaction as the virus is polyvalent, suggesting that the binding of HIV to CCR5 might be cooperative and, therefore, difficult to inhibit. Several lines of evidence support this hypothesis. First, mutations in CCR5 which drastically reduce the affinity of the receptor for gp120 had little effect on the ability of the mutated receptors to support infectivity. Second, immunogold electron microscopy revealed that both CD4 and CCR5 are organized in homogenous microclusters often separated by less than a viral diameter.

The latter observations imply not only cooperative binding, but that following initial interaction with a cluster of CD4 molecules, the virus acts as a tethered ligand greatly enhancing the kinetics of its productive interaction with CCR5. Inhibition of such a kinetically favored, cooperative, interaction necessitates reducing free receptor levels to a minimum.

An effective antagonist must 1) block all states of the receptor to which gp120 can bind, 2) form complexes with the receptor which have very slow dissociation rates, and 3) limit viral access to receptor which just been brought to the cell surface.

391P CHEMOKINE INHIBITORS FROM VIRUSES

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Chemokines play an important role in the immune response to infection and therefore during their evolution pathogens (including viruses) have developed measures to counteract the action of chemokines.

Viruses have developed several different strategies. Some viruses, such as members of the herpes and poxvirus families, express cytokines that bind to chemokine receptors on host cells but do not induce signal transduction by these receptors and thus act as antagonists of the host chemokines. Other chemokines expressed by some herpes viruses are able to both bind and signal via specific chemokine receptors. The specificity of this signalling is presumably beneficial to the virus in the host animal.

Another strategy employed by herpes and poxviruses is to express proteins on the surface of virus-infected cells that are related to the 7-transmembrane chemokine receptors of cells. These molecules might either bind host chemokines and not signal, thereby acting as a sink to soak up host chemokines, or both bind host chemokines and induce signal transduction that is somehow beneficial to virus replication. Lastly, herpes and poxviruses each express soluble proteins that are unrelated to proteins from hosts but which bind chemokines in solution.

These chemokine-binding proteins may bind the chemokine through either the proteoglycan-binding domain or through the chemokine receptor-binding domain. In the former case, after the chemokine is bound to the virus protein, it may still bind the chemokine receptors on leukocytes and stimulate these cells. However, these cells are unable to be recruited to areas of inflammation because the proteoglycan-binding domain of the chemokine is masked by the virus protein. In the latter case, the chemokine is unable to bind to its natural receptors and so leukocytes cannot be activated or recruited, although the chemokine complexed with the virus protein might still bind to proteoglycans on endothelial cell walls in areas of inflammation or elsewhere.

A review of these virus strategies to modulate chemokine activity will be given with emphasis on strategies employed by poxviruses.

392P CHEMOKINES AND CANCER

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We have studied the chemokine receptor expression in breast cancer cell lines and determined that the expression of these receptors is not random but instead is specific. In particular, two receptors were expressed at high levels, CXCR4 and CCR7. The ligands of CCR4, CXCL12, is expressed at high levels in lymph nodes, lung and liver, while the ligand of CCR7, CCL21, is expressed at high levels in lymph nodes. This suggests that these ligand/receptor pairs may play a role in organ-specific metastasis.

To explore this further, we showed that breast cancer cells respond to CXCL12 and CCL21 in vitro, and in vivo an antibody against CXCR4 inhibited metastasis of the MDA-231 breast cancer cell line in a mouse model.

393P CCR3 AS A THERAPEUTIC TARGET FOR ASTHMA AND ALLERGIC DISEASES

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Asthma is a chronic disease of the airways where the inflammatory infiltrate is comprised predominantly of eosinophils. Though preferential increases in eosinophils has been shown to correlate with asthma severity and with compromised expiratory flow and bronchoreactivity, the clinical benefit of inhibiting eosinophil influx into the lung is unclear. Clinical data generated with anti-IL-5 antibodies may provide some insight since blocking IL-5 with antibodies has been shown to completely inhibit allergen induced pulmonary eosinophilia as well as airway hyperreactivity in animals. However, recent clinical studies indicate that inhibition of eosinophils alone will not be sufficient to improve lung function and bronchial hyper-reactivity. The effectiveness of corticosteroids, which are considered the "gold standard" therapy in asthma, may be due to their ability to affect a variety of other mechanisms. CCR3/CCR3-ligand interactions selectively regulate eosinophil trafficking and, as such, represent another key therapeutic target for the relief of inflammation in asthma and allergic conditions.

The advantages that CCR3 antagonism may have over an anti-IL-5 strategy is its potential to block the migration of other allergic cell types. These include a subset of Th2 lymphocytes, basophils and mast cells, which exhibit a chemotactic response to CCR3 ligands *in vitro*. Basophils release histamine and LTC_4 and are a major source of pro-allergic cytokines. CCR3+ Th2 lymphocytes produce IL-4 and IL-5 and co-localize with eosinophils in allergic diseases. Mast cells are implicated in both the early and late phase responses, releasing bronchoconstrictive agents, cytokines, and mucus secretogogues. Another advantage of CCR3 antagonism is its potential to block the activation of pre-existing or residual allergic cells that are poised to release bioactive products into the airway wall. CCR3 has been shown to be the most important chemokine receptor eliciting degranulation of eosinophils *in vitro*. CCR3-dependent degranulation may therefore be significant in the allergic airway, particularly close to tissue compartments, such as the epithelium, known to express high levels of eotaxin. There are also selective increases in expression of CCR3 on bone marrow precursor cells in asthmatics who develop allergen-induced airway.

The numbers of $CD34^+$ cells correlated with eosinophil numbers and with FEV1, suggesting that controlling the influx of progenitor cells may also be required to modulate airway disease. Therefore, despite clinical data with anti-IL-5 antibodies, regulation of CCR3 mediated responses continues to offer the potential for an important therapeutic in the treatment of asthma and allergic disorders.

394P DISSECTION OF INFLAMMATORY EVENTS USING CHEMOKINE ANTAGONISTS: LESSONS FROM TRANSPLANT REJECTION

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While first characterized by their ability to induce the migration of leukocytes, chemokines have subsequently been shown to play roles in leukocyte recruitment, activation, and effector function, as well as hematopoesis, the modulation of angiogenesis, and important aspects of adaptive immunity. These diverse biologic actions can be seen to underlie many of the acute and chronic disease processes that comprise allograft rejection. Aside from the obvious relevance to clinical questions, research into the biology of organ transplantation has been helpful in identifying and dissecting novel aspects of inflammatory processes.

Studies using the targeted disruption of specific chemokines and chemokine receptors, natural receptor mutants, receptor antagonists and blocking anti-receptor antisera, have begun to shed light on the pathophysiological roles of specific chemokine receptors in acute and chronic inflammatory events as they relate to allograft rejection.

Transplant rejection is mediated to a significant degree by the influx of effector monocytes and T cells. The receptor CCR1 is expressed by subpopulations of CD3+, CD4+, CD8+ and CD16+ leukocytes. In transplantation studies, mice lacking CCR1(-/-) show prolongation of cardiac allograft survival (reviewed in 1). In a rat cardiac allograft model a CCR1 blockade using the antagonist BX 471 and sub-therapeutic dose of cyclosporin A was effective in prolonging cardiac allograft survival.

Allograft rejection is thought to be the result of a Th1-type immune response. Th1-like T cells often express CXCR3 and CCR5. Recent studies have emphasized the functional importance of targeting these chemokine receptors in allograft rejection (reviewed in 1). CCR1 and CCR5 share chemokine ligands. In *in vitro* studies using parallel flow chambers it was found that, regardless of the relative level of CCR1 and CCR5 receptor expression, chemokine induced arrest of monocytes and Th1-like T cells was mediated predominantly by CCR1 suggesting specialized roles of apparently redundant receptors in distinct steps of leukocyte trafficking. The importance of CCR5 in human renal allograft survival was demonstrated in a study of patients genetically lacking CCR5. The absence of CCR5 (homozygous CCR5 32) correlated significantly with long term engraftment as compared to the heterozygous or wild type allele, suggesting a role for CCR5 in chronic renal allograft dysfunction.

Antagonists for chemokines and their receptors have the potential to become important therapeutics in treatment of acute and chronic allograft rejection. Therapeutic effects will likely differ depending upon the stage of rejection and the other therapeutics administered.

[1]Nelson, P. J. and A. M. Krensky. 2001. Chemokines, Chemokine Receptors and the Biology of Allograft Rejection. Immunity 14: 377-386

395P APPLICATION OF PROTEOMICS TO THE STUDY OF PHOSPHORYLATION CASCADES

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Three kinds of experiments have been carried out successfully in our laboratories:

(1) Identification of post-translational modifications of the endothelin A and B receptors (ETAR and ETBR) including both phosphorylation and acylation. We have developed new, very efficient methods for single step isolation of highly pure ETAR and ETBR from cells. This has allowed us to obtain evidence that the post-translational modifications are very complex and result in multiple phenotypes showing different forms of modification for receptor. As with other systems, e.g. insulin-like growth factors, it is probable that these multiple phenotypes of the ET receptors correspond to different forms of signalling dependent on cellular state, e.g. the cell cycle. It is, for example, already clear from the phosphorylation of the receptor that a series of different kinases must be involved.

(2) Following stimulation of fibroblasts with endothelin, phosphorylation/dephosphorylation signalling cascades involving several hundred proteins have been observed by use of high resolution 2D electrophoresis and detection of phosphorylated proteins labelled with ^{32}P by autoradiography or immunological methods. The large number of proteins involved are being identified by mass spectrometric methods such as mass fingerprinting or sequencing by mass spectrometry.

(3) Differential gene expression has been followed by using ^{35}S Met pulse chase labelling concurrently with endothelin stimulation. At least 50 proteins showed significant changes in expression on 2D gels and these proteins are also being identified.

These experiments demonstrate that it is now possible to use proteomics methods to investigate the integration of response to an extracellular signal at the levels of the receptor itself, the subsequent signalling cascades and the ensuing gene expression. The proteomics technology permits concurrent monitoring of large numbers of protein phenotypes (the forms and amounts of individual proteins and is therefore able to provide a global overview of signalling processes which greatly augments more traditional investigations of individual proteins or pathways. Furthermore, these new methods will allow quantitative determination of the changes in protein phenotypes, which is very important in view of the highly non-linear amplification properties of such signalling processes.

396P GENETICS AND GENOMICS IN THE DISCOVERY/DEVELOPMENT OF NEW DIAGNOSTICS AND PHARMACEUTICALS: OPPORTUNITIES AND CHALLENGES

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Opportunities:'

- A fundamental improvement of our understanding of pathology on the most basic level
- Shift from clinical to molecular diagnosis: increase, eventually dominant, role of in-vitro diagnostics
- Discovery of new, causal targets allows discovery of more targeted medicines
- New paradigm: predisposition testing – targeted monitoring – early diagnosis – prevention

Challenges:

- An enormous amount of clinical data must be collected and analysed in correlation with genomic/genetic data
- Maintenance of an informed dialogue with a concerned public; fostering the understanding of the probabilistic, not deterministic, nature of all medical data
- Societal consensus on what uses of personal medical information are condoned or disallowed
- Setting appropriate, realistic expectations

397P EXPLOITING GENOMICS AND PROTEOMICS FOR RAPID, EARLY-STAGE DRUG DISCOVERY

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Genomics is revolutionizing the way in which we interpret biology. Downstream technologies such as proteomics and structural biology underpin our understanding of biological structure and function, while genome-wide transcriptome and proteome analysis allow us to gain an impression of the dynamic processes underlying biochemistry and physiology. Nowhere is the potential impact of genomics greater than in the fields of pharmacology and drug discovery.

Until now, reducing genomics to drug discovery practice has centred primarily on the production of protein targets for high-throughput biochemical screening. With bioinformatics allowing the definition of specific gene families and their relationship to biochemical function, many of the members of tractable gene families have already been reduced to discovery practice through screening approaches.

Crucial to our understanding of the inter-relationships between specific targets and their associated gene families has been the development of computational bioinformatics. The most elegant recent example of this is undoubtedly the sequencing and assembly of the human genome (Bailey *et al.*, 2001a) a feat that lay at the very edge of our ability to handle such complex computational tasks. Parallel developments in the area of chemoinformatics, essential for future pharmacological applications, threaten to overwhelm our current computational capabilities.

NMR- and X-ray-based structural biology approaches, themselves early examples of the application of computation to molecular biology, are now providing crucial information in the drug discovery process. The ability to visualize and exploit the detailed architecture of specific active sites has already led to major advances in drug discovery. It is these early successes in structure-based design (SBD) that herald the industrialization of the design process (Dean *et al.*, 2001).

Another key, complementary area of pharmacology is that of ligand-based design (LBD). The way in which both biological and synthetic ligands associate with sites underpins their biochemical activity. In this context, the pharmacophore hypothesis, by reducing complex molecular and spatial information from a set of active molecules to a defined number of points, has been of great value for drug design and development. Massive datasets already exist in the LBD area, and their reduction to practice, in concert with SBD, provides a highly effective way of integrating "lead" discovery. The arrival of *de novo* methods of design extends these technologies to new areas of chemistry-led discovery and lead optimisation (Bailey *et al.*, 2001b)

The presentation will illustrate some of these recent developments in genomics-based drug design and chemoinformatics, and project some of the ways in which they will impact both drug discovery and the shape of pharmaceutical companies of the future.

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Bailey, D.S., Zanders, E. & Dean, P. 2001b The Pharmacogenomics Journal, *1*, 38-47.

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398P OESTROGEN AND BRAIN VULNERABILITY

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Accumulated clinical and basic evidence suggests that gonadal steroids affect the onset and progression of several neurodegenerative diseases and schizophrenia, and the recovery from traumatic neurological injury such as stroke. Thus, our view on gonadal hormones in neural function must be broadened to include not only their function in neuroendocrine regulation and reproductive behaviours, but also a direct participation in response to degenerative disease or injury.

Recent findings indicate that the brain up-regulates both estrogen synthesis and estrogen receptor expression at sites of injury. Genetic or pharmacological inactivation of aromatase, the enzyme involved in estrogen synthesis, indicates that the induction of this enzyme in the brain after injury has a neuroprotective role.

Some of the mechanisms underlying the neuroprotective effects of estrogen may be independent of the classically defined nuclear oestrogen receptors (ERs). Other neuroprotective effects of oestrogen do depend on the classical nuclear ERs, through which estrogen alters expression of oestrogen responsive genes that play a role in apoptosis, axonal regeneration, or general trophic support. Yet another possibility is that non-classical ERs in the membrane or cytoplasm alter phosphorylation cascades, such as those involved in the signalling of insulin-like growth factor-I (IGF-I). Indeed, ERs and IGF-I receptor interact in the activation of PI3K and MAPK signalling cascades and in the promotion of neuroprotection.

The decrease in estrogen and IGF-I levels with aging may thus result in an increased risk for neural pathological alterations after different forms of brain injury.

399P OESTROGEN RECEPTOR-INDEPENDENT MECHANISM FOR NEUROPROTECTION IN STROKE.

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Various lines of evidence indicate that estrogens can protect neurons independently of transcriptional activation through estrogen receptors (ERs). ER binding and transactivation requires stereospecific interaction with ERα, ERβ or a yet undescribed ER. We have assessed estrogen-induced neuroprotection in a hippocampal cell line, which either lacks ERs or expresses levels too low to bind radiolabeled 17βE2.

We analyzed over 40 novel estrogens that vary in ER binding affinity and in agonist/antagonist potency, and found no relationship between binding to ERα or ERβ and neuroprotective effects. Four compounds with low ER binding were compared to 17βE2 in a middle cerebral artery (MCA) occlusion model for cerebral ischemic injury. Equivalent neuroprotection with 17βE2 was observed with each of these compounds: 17 α-E2, a weak estrogen, ent-E2 (the complete enantiomer of 17βE2), and two novel estrogens, ZYC-3 and ZYC-13.

To begin to describe the ER-independent estrogen signaling mechanisms for neuroprotection, we utilized both MCA occlusion and HT-22 cells. To date, we have demonstrated the following: (1) 17βE2 prevents activation of NFκB that is normally seen following ischemia. This inactivation of NFκB by estrogens appears to be related to its ability to prevent the phosphorylation of IκB, a reactive oxygen-stimulated event needed for NFκB activation and (2) in HT-22 cells, 17βE2 activate then inactivate PKCε and ERK 1/2. This activation/inactivation of PKC and ERK signaling by 17βE2 is involved in its neuroprotective activity, as evidenced by the potent neuroprotective effects in HT-22 cells of PKC or MEK inhibitors.

Collectively, these data suggest that neuroprotection can be achieved through mechanisms that are independent of ER binding and subsequent transcriptional activation by estrogens. This provides a novel strategy for drug discovery for brain protection.

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400P ORGANIZATIONAL AND ACTIVATIONAL EFFECTS OF SEX STEROID HORMONES IN THE BRAIN: IMPLICATIONS FOR GROWTH, NEUROPROTECTION AND DISEASE SUSCEPTIBILITY

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Studies on the neural regulation of reproductive functions within the hypothalamus have led to the view that sexual differentiation of the brain arises primarily as a result of exposure to differences in the prevailing levels of circulating sex steroid hormones (SSHs) which occur not only after puberty, but also during a critically sensitive window during development. In particular, a rise in the male sex hormone, testosterone, produced by the testes in late fetal/early neonatal life in rodents, or towards the end of the first trimester in humans, is believed to exert 'organizational' effects that permanently masculinize specific brain regions. Further influences occur in adulthood when SSHs exert reversible 'activational' effects on neuronal activity.

***Hypothalamo-pituitary-growth hormone (HP-GH) axis*:** It is now clear that these dual organizational-activational effects prevail in hypothalamic pathways involved in the regulation of endocrine functions that are not directly associated with reproduction. Notably, there are marked sex differences in the hypothalamic circuitry regulating patterns of GH release which, in turn, determine male/female differences in the rate of postnatal body growth, shape and composition. Our work, focusing on the hypothalamic somatostatin neuronal pathway governing GH secretory patterns, has demonstrated differential activational influences of gonadal factors in adult male and female rats. In addition, certain sex differences are imprinted in early life and, in accord with current views on the mechanisms whereby testosterone masculinizes brain function, they occur as a result of testosterone acting via estrogen receptor-dependent mechanisms after its conversion to estradiol by aromatase enzymes located in the brain. These studies thus address the central mechanisms whereby both neonatal and post-pubertal gonadal SSHs are required for full expression of sex differences in neuroendocrine function.

Extra-hypothalamic brain sites: Numerous recent studies demonstrate that the activational effects of SSHs extend outside the hypothalamus and may contribute to sex differences in the prevalence of certain neurodegenerative conditions. Specifically, Parkinson's disease, which occurs with greater frequency in males than females, is thought to result from the degeneration of the nigrostriatal dopaminergic (NSDA) pathway. Our recent work has defined sex differences in this pathway in gonad-intact adult rats in terms of DA cell numbers, DA content and sensitivity to toxic insults (6-hydroxydopamine [6-OHDA] lesions). In agreement with a growing body of evidence, we find that gonadal factors in the female, *viz* estrogen, are neuroprotective. However, in males, lesions resulting from submaximal doses of the neurotoxin were exacerbated by gonadal factors. Furthermore, physiological levels of estrogen provided no protection against 6-OHDA lesions in males, but may even worsen DA depletion in the striatum in response to the toxin. While it remains to be determined whether these sexually differentiated responses to the activational effects of estradiol in the NSDA are due to the organizational influences of SSHs, these data caution against any assumption that the beneficial effects of estrogen in females may be universally transferable to males.

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401P BRAIN CORTICOSTEROID RECEPTORS AND RESPONSIVE GENES: TARGETS FOR THERAPY OF STRESS-RELATED DISORDERS

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Glucocorticoid hormones (cortisol/corticosterone in man and corticosterone in rodents) secreted by the adrenals in response to stress have profound effects on the brain. These actions exerted by glucocorticoids occur in concert with the hypothalamic-pituitary-adrenal (HPA) axis; they serve to mobilize energy stores and to promote adaptation to change. Yet, when there is either a lack or an excess in glucocorticoids, this very same hormone facilitates breakdown of adaptation, enhances susceptibility to disease and promotes damage typical for stress-related disorders such as e.g. depression. A fundamental question in stress research is therefore how the action of glucocorticoids can change from *protective* to *harmful*. What is the *cause*? What is the *consequence*? (de Kloit *et al.*, 1999)

Two anti-parallel stress systems operate in balance

To address this question we focus on the hormone's nuclear receptors which modulate gene transcription. Two types of receptors exist, which bind glucocorticoids with a ten-fold difference in affinity and are expressed abundantly in hippocampus, a structure involved in mood and cognition. One type, the mineralocorticoid receptors (MR), binds corticosterone, cortisol and aldosterone with high affinity. The other low affinity receptor is the classical glucocorticoid receptor (GR) activated after stress. We found that MR operates in a *pro-active* mode and facilitates immediate behavioural responses aimed *to limit* disturbance of homeostasis. In contrast, GR occupied after stress operates in *re-active* mode and helps to *recover* from homeostatic disturbance and to facilitate behavioural adaptation (Oitzl *et al.*, 2001). The human genome revealed that MR and GR belong to two modes of the stress system organized in antiparallel fashion. One system involving MR is driven by CRH-1 receptors and sympathetic nervous activity (fight/flight), while the matching system promotes coping and adaptation, and depends on the recently discovered stresscopin CRH-2 receptors (Hsu & Hsueh, 2001) and involves GR. The *cost* to maintain balance in these two stress systems (termed *allostatic load*) is thought to enhance disease susceptibility for which the individual is pre-disposed genetically and/or by previous (early) experience (de Kloit *et al.*, 1999).

Stress hormone responsive genes

Nicole Datson found that about 2% of the hippocampal transcriptome (about 700 gene products) appears responsive to MR, GR or both (Datson *et al.*, 2001). Moreover, a differential gene expression pattern was generated by Dorine Feldker (unpublished) from hippocampus of two mouse lines selected for extreme differences in coping ability representing the two anti-parallel stress modes. Many of the gene products we found are unknown, but others appear to belong to genes involved in energy metabolism, structural plasticity, information transfer and ion homeostasis. Under adverse conditions these genes may qualify as *candidate vulnerability genes* and serve as leads for development of novel drugs to treat stress-related brain disorders.

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402P PRENATAL GLUCOCORTICOID PROGRAMMING OF THE BRAIN

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Epidemiological studies have associated low weight or thinness at birth with a markedly increased risk of hypertension, insulin resistance/type 2 diabetes, ischaemic heart disease and affective disorders in adult life. In explanation, the notion of fetal 'programming' has been proposed, but any underlying mechanisms are unclear. Fetal overexposure to stress and its glucocorticoid (GC) effectors, known to reduce birth weight and alter tissue development, might be pertinent

In rats, birth weight is reduced following prenatal exposure to the synthetic GC dexamethasone, which readily crosses the placenta. Similar effects are seen with carbenoxolone (CBX) which inhibits 11ß-hydroxysteroid dehydrogenase type 2 (11ß-HSD2), the physiological feto-placental enzyme 'barrier' to high GC levels in the maternal circulation, thus increasing fetal exposure to endogenous GCs. Variations in placental 11ß-HSD2 activity correlate directly with fetal weight. Whilst the DEX- or CBX-treated offspring regain the weight deficit by weaning, as adults they exhibit permanent hypertension, hyperglycaemia and hyperinsulinaemia.

A potential underlying basis for these pathophysiological effects is increased expression of the nuclear glucocorticoid receptor (GR), noted in liver and adipose tissue, and therefore increased expression of GC target genes. In liver these include PEPCK, the rate-limiting enzyme of gluconeogenesis, which is inappropriately elevated in adults from DEX-exposed pregnancies. Strikingly, in the face of increased tissue sensitivity to GCs, plasma corticosterone (CORT) levels are elevated. Recent work has addressed the potential mechanisms of such HPA activation. These appear to depend upon the timing of exposure to GCs.

Thus, whilst all prenatal models employed show both increased plasma CORT and CRH gene expression in the hypothalamus, effects in higher CNS centres of feedback or HPA drive differ strikingly. Exposure to DEX in the last trimester reduces expression of GR and the higher-affinity mineralocorticoid receptor (MR) in the hippocampus, a key locus of GC negative feedback control upon the HPA axis. In contrast, GC exposure throughout gestation, has no effect upon the hippocampal GR or MR, but increases levels of both receptors in specific nuclei of the amygdala which, in contrast to the hippocampus, activates the HPA axis. CBX, acting largely in the second trimester when the feto-placental 11ß-HSD2 'barrier' is at its maximum, has effects intermediate to these DEX models. These data suggest that whilst HPA activation is a common outcome, the central neuronal pathways underpinning this effect are specific to the stage of exposure.

The amygdala also underpins anxiety and fear behaviour and learning. Prenatal GC exposed rats, as adults show increased anxiety behaviours. The effects correlate with increased CRH expression in the amygdala, but again the specific phenotypes differ somewhat between models, reflecting the particular window of exposure. The molecular mechanism of differential programming of GR in different tissues appears to reflect effects upon multiple tissue-specific alternate first exons/promoters of the GR gene.

In humans, 11ß-HSD2 gene mutations cause very low birth weight and several studies show reduced placental 11ß-HSD2 activity in association with low birth weight/intrauterine growth retardation. Moreover, low birth weight babies have higher plasma cortisol levels and exaggerated GC responses to corticotrophin throughout adult life, indicating HPA axis programming. Indeed, babies exposed to dexamethasone *in utero* show increased 'emotionality' and have higher blood pressures, at least in adolescence. Thus pharmacological or pathophysiological exposure to excess GCs in the prenatal period can programme cardiovascular, metabolic, neuroendocrine and behavioural pathologies in adult life. Whether this is a common or exceptional pathway remains undetermined.

403P NEW STUDIES OF THE PHARMACOLOGY OF PARACETAMOL.

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For the past 5 decades paracetamol (acetaminophen) has been one of the most widely used analgesic/antipyretic agents in medical use – rivalled in popularity only by aspirin and other nonsteroidal antiinflammatory drugs (NSAIDs). Although paracetamol is frequently placed into the NSAID class of drugs because of its antipyretic and analgesic properties, it possesses sharply reduced antiinflammatory activity in comparison to other members of this class. Paracetamol also lacks a carboxyl group that is an important structural feature of most NSAIDs.

NSAIDs act as inhibitors of prostaglandin G/H synthase, which catalyzes the rate-limiting steps in the synthesis of prostaglandins (PGs). The synthase contains two active sites, a cyclooxygenase (COX) active site which cyclizes and oxygenates arachidonic acid to form a short-lived intermediate, PGG_2, and a peroxidase active site that reduces PGG_2 to form PGH_2. All NSAIDs except aspirin act as competitive inhibitors of the COX active site. Aspirin, in contrast, irreversibly acetylates this site. Two isoforms of the synthase are known and are commonly called COX-1 and COX-2. These isozymes show extensive structural similarity but sufficient differences to allow the development of selective COX-2 inhibitors such as celecoxib and rofecoxib. COX-1 is constitutively expressed in cells and tissues, whereas COX-2 is an inducible isozyme.

Paracetamol – tested against intact cells, cell homogenates or purified COX isozymes – demonstrates the paradoxical feature of *stimulating* COX activity at physiologically-relevant concentrations below 1 mM. Only at higher concentrations does it inhibit COX activity. Moreover, paracetamol does not appear to compete with other NSAIDs in binding the COX active site and, therefore, must inhibit COX by a different mechanism.

Using the monocyte/macrophage cell line, J774.2, we have found that we can induce a COX enzyme that is inhibited by sub-millimolar concentrations of paracetamol. This enzyme is likely to represent a sub-population of COX-2 that may differ in redox state and structure from the COX-2 enzyme in inflammatory cells. The inhibition of specific sensitive COX subpopulations provide a plausible mechanism of action for paracetamol.

404P WHAT IS NEW IN PARACETAMOL POISONING?

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Paracetamol poisoning accounts for 48% of hospital admissions for poisoning and approx 200 deaths every year in the UK (Hawton *et al*, 1998). It accounted for 5.5% of poisoning episodes reported to AAPCC TESS scheme in the USA in 1997 (Litovitz *et al,* 1998) It is the commonest reason for transplantation for fulminant hepatic failure in the UK (Bernal & Wendon, 1999)

I will discuss:

The mechanism of paracetamol hepatoxicity

What is new in the management of early paracetamol poisoning?

At risk groups

Chronic alcohol excess and paracetamol overdoses

What is new in late paracetamol poisoning

Reports of liver damage after therapeutic use of paracetamol in children

Prevention of poisoning by reduction of pack size

Adding antidotes to paracetamol tablets

405P BIOCHEMICAL AND MOLECULAR STUDIES OF PARACETAMOL TOXICITY

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Paracetamol (acetaminophen) causes severe centrilobular hepatic necrosis and renal tubular necrosis in both animals and man when administered at suprapharmacological doses. Studies in transgenic animals have confirmed that drug metabolism is an obligatory step in the toxicological process. The drug undergoes direct detoxication to glucuronide and sulphate conjugates but also undergoes bioactivation, by CYP2E1, CYP1A2 and CYP3A4, to a short-lived chemically reactive N-acetyl-p-benzoquinone imine (NAPQI), which is normally detoxified by glutathione conjugation. However, after overdose, the drug metabolism capacity of the hepatocyte is overwhelmed, there is extensive depletion of hepatic glutathione and covalent binding of drug to hepatic proteins.

Both covalent binding and oxidation of protein and nonprotein thiols by NAPQI (E^{o} = 0.978) have been implicated in the hepatotoxicity. The hepatocyte rapidly senses chemical stress, as evidenced by activation of immediate-early genes c-fos and c-jun (Blazka *et al.*, 1996; Kitteringham *et al.*, 2000). There is nuclear binding of the transcription factors AP-1 and Nrf-2 to their specific enhancer elements, including the antioxidant response element (ARE). The ARE is present in the promoters of a number of genes that orchestrate cell defence by increased synthesis of phase II enzymes and antioxidant proteins including γ-GCS, the rate-limiting enzyme for glutathione synthesis. However, the activity of such essential proteins may itself be compromised by chemical modification by NAPQI (Kitteringham *et al.*, 2000), a process that can be reversed by addition of reducing thiols. It is thought that the mitochondria are a primary cellular target in the pathological process (Placke *et al.*, 1987); however, the ultimate mechanism leading to cell death remains elusive. The final stages of hepatocyte death are complex and involve Kupffer cells, infiltrating macrophages (Laskin & Pilaro, 1986)and inter-cellular signalling pathways involving Fas ligand (Zhang *et al.*, 2000), TNF and nitric oxide. Apoptosis may be observed prior to the tissue succumbing to the extensive tissue necrosis, which ultimately results in organ failure and patient death.

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