ACTH ACTION ON THE ADRENALS

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Updated June 1, 2020

ABSTRACT

The adrenocorticotropin hormone (ACTH) is synthesized by the corticotroph cells of the anterior pituitary gland. ACTH, a post-translational product of the proopiomelanocortin protein (POMC), is a 39-amino acid peptide, its sequence being highly conserved in mammals. ACTH binds to the highly specific, for ACTH, melanocortin (MC) 2 receptors (MC2R) located on the surface of adrenal zona fasciculata cells producing cortisol. MC2R belongs to a superfamily of type 1 G protein-coupled receptors. The family of melanocortin receptors includes five members each having characteristic size, tissue distribution, and biological significance. Thus, the MC1R is the principal melanocortin receptor in the skin where it regulates its pigmentation. The MC3R and the MC4R in the central nervous system regulate food intake and energy homeostasis, and knockout animals for these receptors are obese. The MC5R exhibits a wide distribution although its levels in the central nervous system are low. In the adrenal cortex, it induces aldosterone production from the zona glomerulosa cells. ACTH-mediated cortisol synthesis from the *zona fasciculata* cells depends on a large number of factors of the adrenal microenvironment, such as chromaffin and immune cells, adipocytes, and adrenal innervation. Circadian rhythm of cortisol secretion is ensured by the central and peripheral local adrenal clock system. To activate ACTH the MC2R needs the presence of a small trans-membrane protein, the MC2 accessory protein (MRAP). Mutations of this protein result in the type 2 familial glucocorticoid deficiency (FGD) (FGD) syndrome. Type 1 FGD-syndrome is the result of mutations of the MC2R itself. ACTH stimulates cortisol synthesis and secretion by regulating multiple steps in the steroidogenetic pathway including an increase of the number of low-density lipoprotein (LDL) receptors and the cleavage of the side-chain of cholesterol converting it to pregnenolone, the first and rate-limiting step in cortisol production.

INTRODUCTION

It is now more than 80 years since Selye introduced the concept of “general adaptation syndrome”, later renamed “stress syndrome” as a state of threatened homeostasis in response to stressful stimuli, the stressors (1). Selye was also the first to describe “corticoids” and to propose that glucocorticoids and mineralocorticoids regulated not only carbohydrate and electrolyte metabolism, respectively, but also exerted anti- or pro-inflammatory effects. By stimulating adrenal corticosteroids synthesis, the adrenocorticotropin hormone (ACTH), which was first isolated in 1943 and synthesized in the 1970s, plays a central role in homeostasis and stress and is a key component of the hypothalamic-pituitary-adrenal axis (HPA) axis (2, 3, 4).

The corticotroph cells of the anterior pituitary synthesize and secrete the ACTH which via the circulation binds and activates its receptors in the adrenal fasciculate cells affecting most steps in the synthesis of cortisol. This widely accepted model has been extensively advanced and enriched during the last few years. More specifically, it has been found that for the ACTH receptor, the melanocortin receptor 2 (MC2R), needs the presence of a small trans-membrane protein, the MC2 accessory protein (MRAP) to respond to ACTH. Mutations of this protein result in the type 2 familial glucocorticoid deficiency (FGD) syndrome. Type 1 FGD is the result of mutations of the MC2R itself. Newer data reveal the role of the autocrine-paracrine micro-regulation of ACTH-mediated cortisol synthesis by a large number of intra-adrenally produced factors deriving from chromaffin cells, resident immune cells, intra- and peri-adrenal adipocytes, and adrenal innervation. Moreover, there is an increasing interest in the role of a central and peripheral (endogenous) adrenal clock system exerting a circadian regulation of ACTH secretion and action (5, 6). Great progress has been also made in our understanding of the pathophysiology of the triple A syndrome, which is caused by mutations in the gene encoding the regulatory protein ALADIN, a product of the ADRACALIN gene. The updated version of this chapter includes the classical data regarding ACTH-induced cortisol production by the adrenal gland, as well as a description of the new findings.

ACTH AND ITS PRECURSOR MOLECULE PRO-OPIOMELANOCORTIN (POMC)

The ACTH hormone is the primary regulator of cortisol production synthesized in the human adrenal fasciculate cells. ACTH is a post-translational product of the proopiomelanocortin protein (POMC), which is synthesized in the corticotroph cells of the anterior pituitary gland. ACTH is a 39-amino acid peptide. Its sequence is highly conserved in mammals since only amino acids 31 and 33 vary between higher mammals and primates. The biological activity of the ACTH molecule depends on its first 24 amino-terminal amino acids while fragments of less than 20 amino acids long are completely inactive. However, the residue 25-39 is important for the stability of the molecule, increasing its otherwise short half-life. Truncation of ACTH from the C-terminus gradually reduces its activity while removal of the four basic residues (Lys–Lys–Arg–Arg) in positions 15–18 inactivates it completely. Finally, it should be noted that its first 13 residues activate all melanocortin receptors in addition to the ACTH receptor. ACTH acts through the formation of cAMP which facilitates the transfer of cholesterol into the mitochondrial inner membrane for the synthesis of adrenal steroids (7,8) (Figure 1).

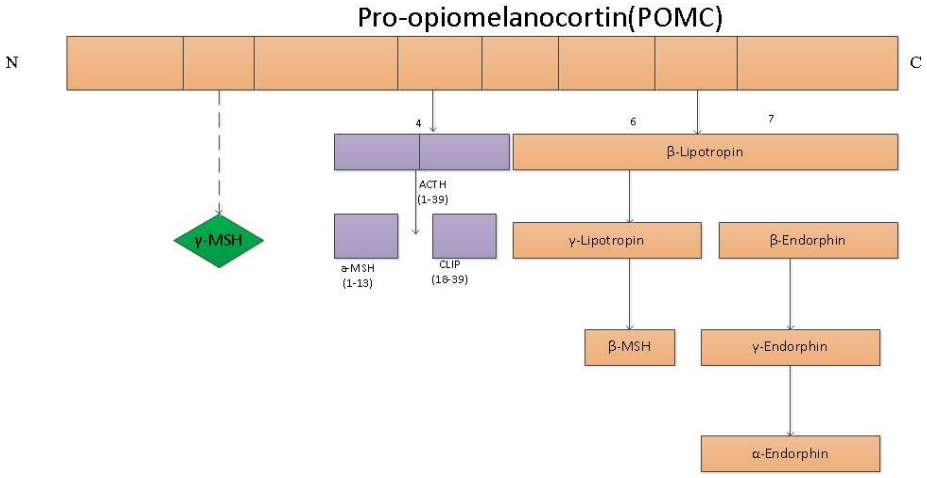

Figure 1. POMC products after enzyme-mediated cleavage.

The synthesis of POMC, its post-translational modification and the secretion of ACTH are under the control of corticotropin-releasing hormone (CRH or CRF) and to a lesser degree to arginine vasopressin (AVP). Both these hormones are synthesized in the parvocellular cells of the paraventricular (PVN) hypothalamic nucleus and are under the negative control of the circulating glucocorticoids. It should be noted that the AVP derived from PVN follows a distinct regulatory and secretory path, completely different from that of AVP synthesized in the magnocellular cells and transferred and secreted from the posterior pituitary as a regulator of water balance. Indeed, the magnocellular-derived AVP is transferred to posterior pituitary by axonal transport and its synthesis and secretion are under the influence of osmotic and oncotic stimuli and plays no part in stress response. On the other hand, the parvocellular-derived CRH and AVP travel, via axonal transport, to the median eminence (ME) at the lower part of hypothalamus from where they are both secreted into the vascular connection between hypothalamus and anterior pituitary, the portal circulation. Multiple neural signals regulate the synthesis of CRH and AVP as well as their secretion from ME.

In addition, a complex central clock synchronized by light information received *via* the retino-hypothalamic tract from the eye, is located in the suprachiasmatic nucleus (SCN) and sends circadian oscillatory stimuli to the PVN, influencing the secretion of CRH and AVP, and generating the circadian secretion of ACTH (9). This central clock entrains the peripheral oscillators in the adrenal gland via three pathways: (a) the neurohumoral pathway via the HPA axis, (b) the neural pathway via the autonomic nervous system, and (c), a local circadian intra-adrenal regulation of ACTH action (10). However, besides the central circadian regulation of ACTH secretion, local adrenal clocks are thought to regulate also the responsiveness of the adrenal cortex to ACTH in a circadian fashion. Moreover, it is known since the 1960s that adrenals tissues can exhibit an intrinsic rhythmicity of cortisol secretion independently of the upstream rhythm of the HPA axis (10). (Figure 2)

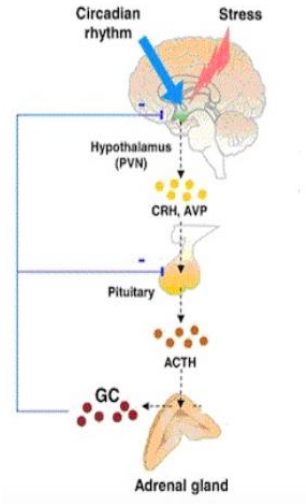

Figure 2. The pathway of stimulation of ACTH secretion from the pituitary and its action on the adrenal gland (6).

CRH reaching the anterior pituitary corticotrophs and binds to the CRH-R1 receptors. The corticotrophs represent approximately 10% of anterior pituitary cells. Their main product, POMC is a 260 AA protein,

which is post-translationally cleaved into several bioactive peptides that are secreted from the corticotrophs along with ACTH, including β-lipotropin, the endogenous opioid peptide beta-endorphin, and melanocyte stimulating hormones (MSH) (11,12).

Glucocorticoids exert their negative feedback control on both the hypothalamus at the PVN and anterior pituitary corticotrophs suppressing POMC synthesis and ACTH secretion. Furthermore, chronic exposure to high levels of endogenous or exogenous glucocorticoids results in characteristic corticotropic cell degeneration. The immune system participates in the regulation of ACTH production via interleukins (IL)-1, IL-6, tumor necrosis factor (TNF)-alpha and the interferons alpha and gamma which affect the axis at all its levels i.e. hypothalamus, pituitary, and adrenal cortex (13). Finally, the intra-adrenal production of cytokines appears to play an important modulator of the ACTH-mediated effect on adrenocortical cells (14).

EFFECTS OF ACTH ON ADRENAL CORTICAL CELLS

ACTH enters the systemic circulation and binds to the highly specific, for ACTH, MC2R located on the surface of adrenal cortical cells. The adult mammalian adrenal cortex is composed of three zones. The outermost or zona glomerulosa produces aldosterone, the middle or zona fasciculata is the largest producing cortisol, while the innermost or zona reticularis produces the weak adrenal androgens. ACTH is the main stimulus of the zona fasciculata and zona reticularis, stimulating glucocorticoid secretion, while angiotensin II and potassium are the main stimuli of aldosterone secretion by the zona glomerulosa. Most MC2R are localized in the zona fasciculata. In general, the steroids produced by the adrenal cortex are classified as 21-carbon steroids (glucocorticoids and mineralocorticoids), as 19-carbon steroids (adrenal androgens), and 18-carbon (adrenal estrogens). Cortisol, the main endogenous glucocorticoid, is synthesized in zona fasciculata under the exclusive regulation of ACTH. ACTH is of secondary importance in aldosterone production (where plasma angiotensin II and serum potassium represent the main regulators). The production of adrenal androgens is more complicated with ACTH playing a minor role. Under normal circumstances, ACTH acts with equivalent potency as a secretagogue for cortisol and aldosterone. Recently, novel evidence suggested that aldosterone secretion stimulated by ACTH via its receptor [called melanocortin receptor 2 (MC2R)] is observed in adrenal tissues of patients with primary aldosteronism (15).

The mechanism of ACTH action follows the classical peptide hormone rules. Indeed, ACTH binds to its receptor, MC2R, located on adrenocortical cell membranes activating a Gs-protein resulting in an increase of intracellular cyclic adenosine monophosphate (cAMP). cAMP relays ACTH-mediated functions via the activation of the serine–threonine kinase cAMP-dependent protein kinase A (PKA) or the exchange proteins directly activated by cAMP (EPAC1 and 2 also named cAMP-regulated guanine nucleotide exchange factors (cAMP-GEFs) I and II). Following cAMP activation, PKA and EPAC transmit signals differently. PKA phosphorylates numerous substrates, while EPACs act as GEFs catalyzing the conversion of the small GTPases Rap1 and Rap2 from an inactive (GDP-bound) to an active form [guanine triphosphate (GTP)-bound] (16).

ACTH stimulates cortisol synthesis and secretion by regulating multiple steps in the steroidogenetic pathway. Steroid hormones are produced from the same precursor, cholesterol, by a set of cytochrome P450 steroid hydroxylases (CYP11A1, CYP11B1 and CYP11B2, CYP17 and CYP21) and the steroid dehydrogenase 3βHSD (16). The enzymes are differentially expressed in the three zones of the adrenal cortex (zona glomerulosa, zona fasciculata, and zona reticularis) giving rise to zone-specific

hormone production. In humans, the primary source of cholesterol for steroid hormone production is LDL-cholesterol, which is imported via the LDL receptor (LDLR) from the blood stream. About 80% of the cholesterol needed for the synthesis of adrenal steroids is supplied by LDL. ACTH increases the number of LDLR resulting in an increase in overall uptake of cholesterol ester. Once cholesterol ester enters the cell, hormone-sensitive lipase (HSL) converts it to free cholesterol (15). Free cholesterol is then delivered to the inner mitochondrial membrane by the actions of steroidogenic acute regulatory protein (StAR) and cholesterol-binding proteins (17).

The precursor cholesterol for steroidogenesis can be derived from a combination of sources: (1) *de novo* cellular cholesterol synthesis, (2) mobilization of cholesterol's esters (CE) stored in lipid droplets (LD), and (3) lipoprotein-derived CE delivered through endocytic uptake, which is mediated by the LDL receptor or "selective" cellular uptake of HDL cholesterol *via* the scavenger receptor, class B type 1 (SR-B1) (17). Acute and chronic ACTH stimuli can modulate SR-B1 function, resulting in changes in the ability of SR-B1 to mediate cholesterol uptake and use for steroids production. ACTH also regulates the formation of microvillar channels in the plasma membranes which retain HDL particles and contain high numbers of HDL receptors.

Once PKA is activated, both an acute and a chronic response occur, which contribute to increased steroid hormone synthesis. During the acute response, PKA phosphorylates HSL, which converts cholesterol esters to free cholesterol. This rapid response also involves an increase in StAR, which facilitates the movement of cholesterol to the inner mitochondrial membrane, where the limiting enzyme CYP11A1 resides (18). The chronic response corresponds to the transcriptional activation of all the other steroidogenic enzymes.

ACTH affects the cleavage of the side-chain of cholesterol converting it to pregnenolone, the first and rate-limiting step in cortisol production. The CYP11A1 gene which encodes the cholesterol side-chain cleavage is regulated by ACTH and by the steroidogenic factor 1 (SF-1). Moreover, ACTH hydroxylates the pregnenolone in the 17-OH position which is subsequently converted into 11-deoxycortisol. 11-deoxycortisol moves back to mitochondria where a hydroxylation at position 21 results in cortisol which is then rapidly secreted into the systemic circulation (19).

Activation of the MC2R by ACTH in the adrenals also induces the adrenal production of factors affecting adrenal growth and its blood flow. Thus, among other things, ACTH stimulates the intra-adrenal production of vascular endothelial growth factor (VEGF) and the vaso-relaxant epoxy-eicosa-trienoic acids (EETs) (20, 21).

Finally, chronic exposure of adrenocortical cells to high levels of ACTH (from eutopic or ectopic production) results in the development of adrenal hyperplasia, nodules, and finally neoplasia. Activation of ACTH receptor and PKA are considered vital for maintaining the highly differentiated cellular phenotype of adrenal cells and the subsequent activation of ERK is of low importance for cell proliferation. In addition, ACTH signals inactivate Akt, a kinase that promotes survival and proliferation. On the other hand, ACTH receptors are up-regulated in adrenocortical adenomas of patients with ACTH-dependent hyper-cortisolemia, intensifying the adrenal response to the already elevated ACTH, aggravating their disease. ACTH also up-regulates the human homolog of Diminuto/Dwarf1 gene, which is associated with benign adrenocortical adenomas. Low expression of this gene correlates with apoptosis, indicating that its intensified expression may contribute to cell survival.

Wnt-signalling is the main pathway controlling cortisol secretion. Recent studies have shown that cortisol-producing neoplasms frequently display somatic or germline mutations that affect proteins of the cAMP/PKA pathway, leading to constitutive activation of PKA. These mutations include gain-of-function mutations of the *MC2R, GNAS,* the catalytic subunit α of protein kinase A *(PRKACA)* and *PRKACB* genes and inactivating mutations of the regulatory subunit R1α of PKA (*PRKAR1A*), and of two cAMP-binding phosphodiesterases (*PDE11A* and *PDE8B*) genes, mimicking the action of ACTH to stimulate glucocorticoid production, providing a molecular basis for the pathogenesis of primary adrenal Cushing syndrome (22).

It has also been shown that cortisol secretion by adrenocortical adenomas and hyperplasias could be stimulated by both locally produced ACTH (23) and aberrantly expressed membrane receptors, such as those of serotonin (24). Prolonged activation of the cAMP/PKA pathway by ACTH induces an aberrant serotonergic stimulatory loop in the adrenal cortex that likely participates in the pathogenesis of corticosteroid hypersecretion.

Mutations of the *PRKAR1A* are considered the main cause of familial and sporadic primary pigmented nodular adrenocortical disease (PPNAD). Moreover, inactivation of *PDE11A* and *PDE8B* are associated with isolated micronodular disease (iMAD) which can be also found in PPNAD and primary bilateral macronodular adrenal hyperplasia (PBMAH) cases. Recently, a germline mutation in Armadillo repeat containing protein 5 (*ARMC5*) gene was found in 25–50 % of PBMAH patients (25). PBMAH that results from *ARMC5* mutations have been shown to contain clusters of ACTH-producing cells that stimulate cortisol secretion in an autocrine/paracrine fashion in adrenal tissues through the MC2R (23).

The role of ACTH in adrenocortical tumors remains to be elucidated. It may depend on the state of differentiation of the particular cell or the presence of additional events that may decide the direction of the ACTH signal towards cell survival or inhibition of proliferation (26,27).

MELANOCORTIN 2 (MC2), THE ACTH RECEPTOR

ACTH exerts its effects on the adrenals via a highly selective receptor, a member of the MC2R superfamily of type 1 G protein-coupled receptors. As mentioned above, the MC2 receptor is highly specific for only one ligand, ACTH (28). The family of melanocortin receptors includes five members, each having characteristic size, tissue distribution, and biological significance (29). The MC system and its receptors regulate multiple physiological processes including skin pigmentation, glucocorticoid production, food intake and energy balance. The MC2R is a 297 amino acid transmembrane G-protein coupled receptor. In humans, it maps to 18p11.2. Activation of the MC2R initiates a cascade of events affecting multiple steps in adrenal cortisol production. The MC2R is dependent on a small accessory protein, melanocortin receptor accessory protein (MRAP), which is essential for both trafficking of MC2R to the plasma membrane and for ACTH binding and activation of MC2R. MC2R–MRAP interactions may affect the trafficking of certain receptors to the cell membrane or allow activation of the receptor by its ligand. Specific mutations in the region of the N-terminal tail of MRAP1 and MRAP2 are essential for promoting only the trafficking of receptors to the plasma membrane (MRAP2) or essential for ACTH-MCR2 receptor ligand recognition and function (MRAP1). (see below).

Mutations in the MC2 may result in familial glucocorticoid deficiency, a group of autosomal recessive disorders characterized by resistance to ACTH. It should be noted that although the MC2R is expressed predominantly in the adrenal cortex, it is also present in skin melanocytes where its ligand ACTH also binds to the MC1 thus affecting skin

pigmentation. Indeed, chronically elevated ACTH in the circulation (chronic adrenal insufficiency or ectopic ACTH production or in Nelson's syndrome following adrenalectomy) can induce skin and gum hyper-pigmentation. MC2R is also expressed in adipocytes and mediates stress-induced lipolysis via central ACTH release. The MC2R is localized in all three zones of the adrenal cortex. Results from binding studies indicate that in the adrenal cortex MC2R can be subdivided into a type with a KD of 1 nM, but with only 60 binding sites per cell and into a second type with a KD of 300 nM, but with several orders of magnitude more binding sites (about 600,000) per cell. The presence of high and low affinity receptors for ACTH means that the adrenal cortex is highly sensitive and specific to the usual concentrations of ACTH in the systemic circulation (30).

Intra-Adrenal Regulation of Cortisol Production

The fasciculate cells of the adrenal cortex are affected by multiple factors produced within the adrenal gland. It should be noted that in addition to steroidogenic cells, the adrenals contain the chromaffin cells in the adrenal medulla arranged in columns crisscrossing the length of the gland, nerve fibers from intra- and extra-adrenal neurons, multiple cells of the immune system including monocytes / macrophages, mast cells, lymphocytes, vascular endothelial cells, and adipocytes within and around the gland. All these cells form complex intra-adrenal networks of interaction affecting, among other things, the response of fasciculata cells to ACTH, the expression of the MC2 receptors and their associated proteins, the growth and vascularization of the gland and many other functions. In addition, it has been also shown that the adrenal glands exhibit an intrinsic rhythmicity of corticosterone secretion in animal studies. Indeed, adrenal denervation leads to an abolishment of both the circadian corticosterone rhythm, as well as of the daily variation of the adrenal responsiveness to ACTH (9).

Role of Adrenal Chromaffin Cells

Chromaffin cells in the adrenal medulla originate from neural crest, their main products being the catecholamines epinephrine and norepinephrine. Chromaffin cells also produce neuropeptides and cytokines released together with catecholamines. Chromaffin cells are not clearly separated from the adrenal cortex as previously thought. Indeed, chromaffin cells can affect adrenal cortical cells in a paracrine mode of action since they can be found in all zones of the adult adrenal cortex up to the outer layer of the cortex i.e. zona glomerulosa and may form larger conglomerates of chromaffin in the adrenal subcapsular region. On the other hand, cortical cells are also located in the medulla, where they may form islets surrounded by chromaffin cells. This close association between cortical and chromaffin cells allows a paracrine regulation of adrenocortical steroidogenesis. Indeed, adrenal chromaffin cells synthesize a multitude of neuropeptides including beta-endorphin, the enkephalins, the dynorphins, CRH, substance P, adrenomedullin, neuropeptide Y (NPY), vasoactive intestinal peptide (VIP), atrial natriuretic peptide (ANP), somatostatin etc. These neuropeptides can affect either the response of cortical cells in zona fasciculate to ACTH, the vascularization of cortex, its growth, or they may exert direct modulatory effects on the cortical cells themselves. These effects on the adrenal cortex coordinate the two stress axes and streamline steroidogenesis as per the needs of the adaptation response to stressful stimuli (31-35). (Figure 3).

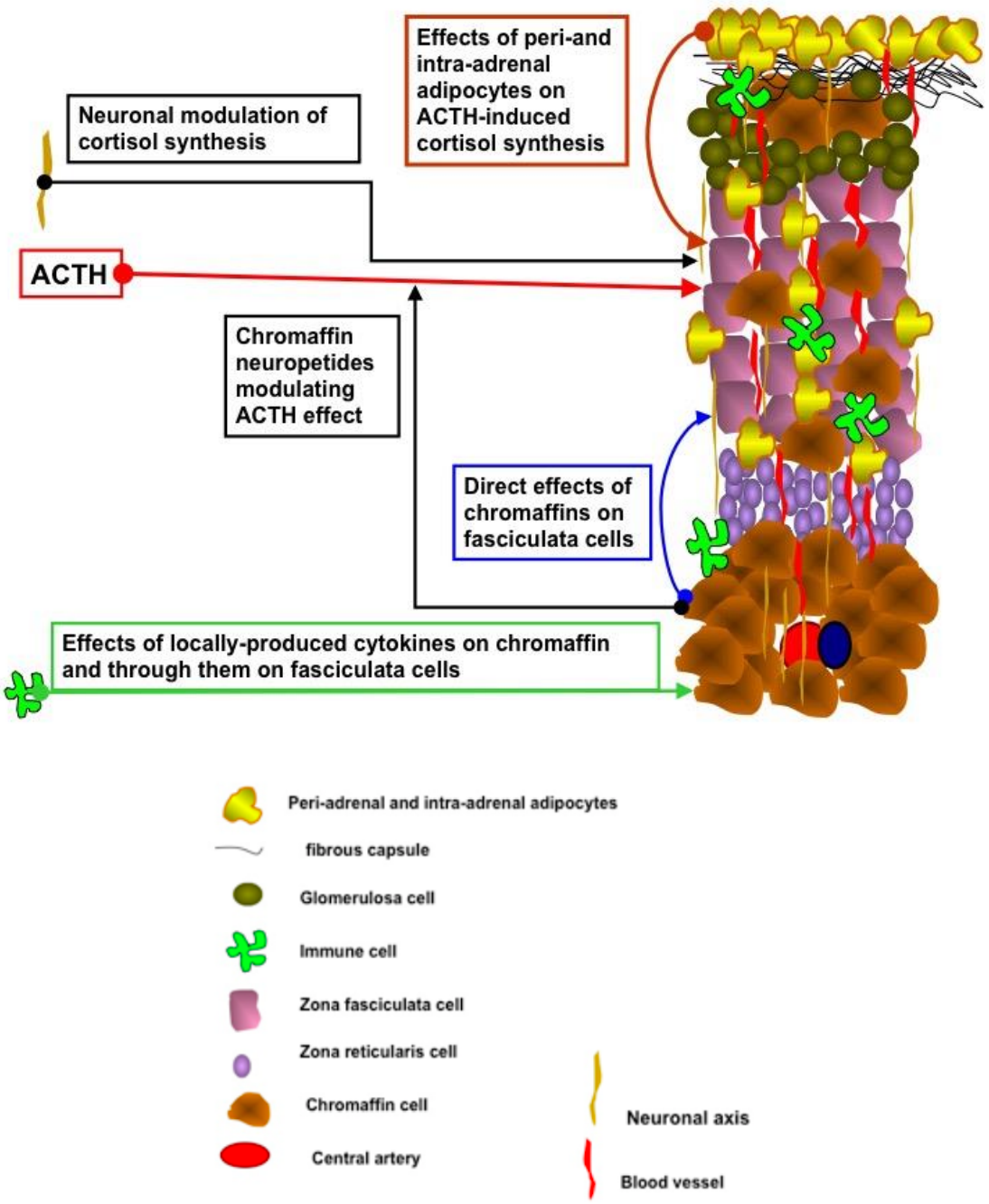

Figure 3. Autocrine and paracrine effects of intra-adrenally-produced substances on ACTH-induced cortisol synthesis.

Role of Peri- and Intra-Adrenal Adipocytes on the Adrenal Effects of ACTH

It is now suspected that the peri- and intra-adrenal adipocytes modulate the effects of ACTH on adrenocortical cells. Thus, it has been shown that leptin exerts an inhibitory effect on ACTH-induced corticosteroid production by human adrenocortical cells without affecting their viability and proliferation. It should be noted that murine adipocyte cell lines

and immortalized adipocytes express the MC2R suggesting that these adipocytes are also affected by ACTH (36-37).

THE MC2 SIGNALING PATHWAY

As stated above, the MC2R is a G-protein coupled receptor. Among the G proteins Gs and Gi2 are implicated in ACTH signalling. ACTH also increases the transcription of G-alpha/q or G-q/11, a hetero-trimeric protein, which couples with the MC2R. G-q/11 activates the phospholipase C pathway. Mutations of the alpha subunits of Gs and Gi2 are associated with adrenocortical tumor formation. Signals that initiate from the MC2R and the G-proteins lead to cAMP formation and activation of PKA and PKC. As a result, several intermediate molecules are involved including kinases and transcription factors that orchestrate the ACTH actions on adrenal cells. The MC2R is a weak activator of MAP Kinases ERK1 and ERK2. ERK1 and ERK2 activation is important in ACTH-triggered mitogenic effects. In normal adrenal cortical cells, MC2 signals lead to activation of the Stress Activated Protein Kinase (SAPK) JNK. Activation of JNK depends on PKC activity and mobilization of intracellular Ca++ implying that both PKC activation and Ca++ influx result from the binding of ACTH to its receptor. In tissue culture experiments using the Y1 adrenocortical tumor cell line, ACTH exerts an antiproliferative effect, mediated by cAMP. ACTH signals result in dephosphorylation and inactivation of Akt/PKB kinase thus inhibiting the proliferation of adrenocortical tumor cells. Such anti-proliferative effect is most likely associated with increased steroidogenesis and suppression of the malignant phenotype of this particular cell line. The MC2R effects are mediated via activation of the cAMP pathway, which includes the cAMP-dependent transcription factors CREM (cAMP responsive element modulator) and CREB (cAMP responsive element binding protein) that result in transcriptional activation of steroidogenic enzymes, cell proliferation and differentiation. Activation of the MC2R leads to stimulation of Fos and Jun transcription, which by heterodimerizing form the AP1 complex. It should be noted here that the *Fos* gene family consists of four members, *c-Fos, FosB, Fra1* and *Fra2,* while the *Jun* family consists of three members, *c-Jun, JunB and JunD*. These proteins form hetero- or homo- dimers inducing transcription through binding to AP1-binding sites. Activation of AP1-dependent transcriptions leads to the production of several pro-mitotic proteins while its inhibition results in a blockade of cell cycle to the G1 to S phase transition.

THE MELANOCORTINERGIC SYSTEM

Conceptually, the fact that the ACTH receptor belongs to the melanocortin receptor family implies a close association between several physiological processes including stress, homeostasis, regulation of food intake and regulation of energy balance, immunity, and skin function. Indeed, ACTH can bind receptors in melanocytes, adipocytes, mononuclear/ macrophages and several areas within the central nervous system, with a much lower affinity compared to that of the MC2R. However, direct actions of ACTH through the MC2R have also been reported in several peripheral tissues. For instance, ACTH inhibits leptin secretion from adipocytes via the MC2R present in adipocytes, an affect indirectly contributing to the regulation of energy homeostasis during stressful periods (38).

The melanocortinergic system in the central nervous system consists of the endogenous agonists alpha-, beta-, and gamma-MSH (post-translational products of POMC), the naturally occurring antagonists, the agouti-related protein (AGRP) produced by the arcuate nucleus neurons in hypothalamus and the agouti protein found in the skin. The AGRP antagonizes alpha-MSH in the hypothalamus at the level of MC3 and MC4R. The agouti protein and AGRP require the presence of a third protein, Mahogany, to antagonize MSH. Mahogany protein is

widely expressed and it is a close relative of Attractin, an immunoregulatory protein made by human T lymphocytes.

Activation of the central melanocortin receptors (MC3 and MC4) by alpha MSH inhibits feeding and alters the rate of energy consumption leading to weight loss, whereas its blockade results in obesity. Development of MC3 and MC4 knockout mice revealed differential actions of each receptor. MC4 -/- mice were hyperphagic with partially increased metabolic efficiency while MC3 -/- animals developed obesity due to increased metabolic efficiency, thus underlying their significance in metabolism and obesity. The MCR is also involved in the regulation of autonomic nervous system tone and of arterial pressure at the level of the central nervous system. The MC receptor appears to be also involved in several higher learning processes. Outside the central nervous system, the MC4 receptor is expressed in osteoblasts where it may be involved in bone remodeling facilitating the communication between osteoblasts and osteoclasts (39-41) (Table).

The MC1 receptor (MC1R) is a 315 amino acid transmembrane protein which in humans is mapped to 16q24. It is the principal melanocortin receptor in the skin where it regulates its pigmentation. It exhibits high affinity for most MSH isoforms and a much lower affinity for ACTH. Its highest affinity is towards alpha–MSH (Ki = 0.033 nmol/l). Stimulation of MC1R in the skin and the hair follicles by alpha-MSH results in induction of melanogenesis producing dark skin and hair in several species including the humans. The MC1R is also present in the adrenals, the leukocytes, lungs, lymph nodes, ovaries, testes, pituitary, placenta, spleen and the uterus. The agouti protein is an endogenous antagonist of alpha-MSH at the level of the MC1R in the skin. Over-expression of the agouti protein results in fair skin, reddish hair and disturbances of energy balance. Variants of the MC1R in humans are associated with red hair, pale skin, and increased risk for skin cancer. The MC1R in leukocytes and macrophages has been associated with the immune effects of alpha-MSH (42).

The MC3 receptor (MC3R) is expressed mainly in the brain. In humans it is a 360 amino acid protein and maps to 20q13.2. The MC3R and the MC4R in the Central Nervous System regulate food intake and energy homeostasis. Knockout (KO) animals for these receptors are obese. The MC4R KO mice are hyperphagic while the MC3R KO animals are not hyperphagic but still obese signifying the effect of this receptor on the overall energy homeostasis. The agouti and the agouti-related protein are endogenous natural antagonists of the MC1R, MC3R and MC4R. Finally, the MC3R may be involved in the mechanism turning off the inflammatory response mainly via suppression of macrophage migration. In the brain the MC3R is mainly expressed in the arcuate nucleus at the basis of hypothalamus where it regulates hunger and satiety.

The MC4 receptor (MC4R) is a 332 amino acid trans-membrane protein. It is expressed in the central nervous system (mainly in the hypothalamus), the gastrointestinal tract and the placenta. In humans, it maps to 18q22. The MC4R is a major regulator of food intake. Inactivating mutations of MC4R cause obesity both in mice and humans. Global homozygous deletion of MC4R in mice results in hyperphagia, increased fat and lean mass, increased body length, reduced activity, and a suppressed metabolic rate. Inactivating mutations in MC4R are the single most common form of monogenic obesity in humans. Common variants near the MC4R locus are associated with adiposity, body weight, risk of obesity, and insulin resistance. In addition to the homeostasis of energy and thermogenesis the MC4R receptor plays other roles including regulation of autonomic control of blood pressure. Finally, the MC4R plays an important role in the production of the

neuropeptides YY and glucagon-like peptide 1 by the enteroendocrine cells (43-47).

The MC5 receptor (MC5R) is a 325 amino acid trans-membrane protein. It is expressed in the adrenals, skin, stomach, lung and spleen. Its levels in the central nervous system are very low. In the adrenal cortex, it is expressed in all three layers but predominantly in the aldosterone-producing zona glomerulosa cells. The presence of MC5R expression in zona glomerulosa may be involved in melanocortin-induced aldosterone production. In the skin, the MC5R affects exocrine function. It is expressed in peripheral lymphocytes and in splenocytes indicating that this may be the receptor utilized by ACTH in those cells. MC5R is expressed in articular chondrocytes mediating cytokine production in the inflamed joints in rheumatoid arthritis. The MC5 receptor also mediates the production of IL-6 from adipocytes contributing to metabolic inflammation and insulin resistance. Indeed, stimulation of the MC5R in 3T3-L1 adipocytes with αMSH induces lipolysis and suppresses re-esterification of fatty acids through the ERK1/2 pathway.

Table. The Melanocortin Receptor Family

Receptor	Ligand affinity	Main site of expression Primary Function	Primary Function	Disease phenotype with loss of function mutations
MC1R	αMSH =ACTH>βMSH > γMSH	Melanocytes	Pigmentation, inflammation	Increased risk for skin cancer
MC2R	ACTH only	Adrenal cortex	Adrenal steroidogenesis	FGD
MC3R	γMSH> αMSH =βMSH>ACTH	CNS, GI tract, Kidney	Energy homeostasis, inflammation, food intake	Obesity
MC4R	αMSH = βMSH = ACTH >> γMSH	CNS	Energy homeostasis, thermogenesis, appetite regulation, erectile	Obesity
MC5R	αMSH > ACTH>βMSH> γMSH	Lymphocytes, exocrine cells	Exocrine function, regulation of sebaceous glands	Decreased production of sebaceous lipids in mice

Abbreviations: MSH: Melanocyte-stimulating hormone, CNS: central nervous system, GI: gastrointestinal tract, FGD: familial glucocorticoid deficiency.

Regulation of MC2 Receptor Gene Expression

The MC2R gene has one untranslated exon (exon one), an 18kb intron, and the coding exon (exon two). The existence of different MC2 transcripts in human adrenal cortical cells suggests the presence of multiple transcription initiation sites. An alternate exon 1 (exon1f) is transcribed in adipose tissue but not in the adrenals. This exon appears to be transcribed by a different promoter region from that

reported in the adrenal, thus conferring tissue specificity. Studies on the MC2 promoter polymorphism reveal a single nucleotide polymorphism close to the transcriptional initiation site (-2C/T) resulting in inhibition of transcription causing reduced MC2 levels even in the heterozygous state. This allele is present in 10% of the population.

The MC2R promoter contains binding sites for several transcription factors. Transcription factors are nuclear proteins modifying the expression of genes by binding to specific DNA sequences usually located upstream of gene promoters. Phosphorylation of a transcription factor results in its activation and modulation of the transcriptional activity of a promoter containing response elements for the specific factor (48).

Factors Affecting the Expression of MC2 Receptor Gene

EFFECTS OF ACTH ON THE EXPRESSION OF MC2R

Several studies have shown that the MC2R gene is up regulated by its own ligand, ACTH. Indeed, ligand-induced up-regulation of MC2R expression may be a crucial adaptive process directed towards optimizing adrenal responsiveness to ACTH. The effect of ACTH on MC2R expression is dependent on cAMP and probably mediated through AP-1(49).

EFFECTS OF GLUCOCORTICOID REGULATORY ELEMENTS (GRE) ON THE MC2R GENE

Glucocorticoids are major regulators of MC2 expression. Glucocorticoids exert an enhancing effect on basal, ACTH- and cAMP-induced MC2 expression.

Steroidogenic Factor-1 (SF-1) is an orphan nuclear receptor. The MC2R gene contains three SF-1 binding sites in the proximity of the transcription initiation site. In addition to its effect on the transcription of the MC2R gene, SF-1 also affects the transcription of genes involved in steroidogenesis in the adrenals and the gonads as well as the organogenesis of both glands. SF-1 knockout mice lack adrenal glands and gonads. SF-1 is also essential for the compensatory adrenal growth following unilateral adrenalectomy. In steroidogenesis, SF-1 affects the transcription of CYP11A1 gene which encodes the P450scc cholesterol side-chain cleavage enzyme, the first step in steroidogenesis. Several SF-1-binding sites on the promoter of CYP11A1 modulate its transcription rate (50).

DAX-1 (Dosage-sensitive sex reversal, Adrenal hypoplasia congenital critical region on the X chromosome, gene 1) is a transcription factor expressed in the adrenal gland and gonads. DAX-1 encodes an orphan member of the nuclear hormone receptor super family. DAX-1 inhibits SF-1-mediated steroidogenesis while its absence augments the adrenal responsiveness to ACTH most probably through an up-regulation of the MC2R transcription via SF-1. A cAMP-dependent PKA augments the SF-1-mediated induction of steroidogenesis. Generally speaking, DAX-1 is a suppressor of the transcription of several genes involved in the steroidogenic pathway. Indeed, inactivating mutations of DAX-1 results in the X-linked form of adrenal hypoplasia congenital (AHC) with associated hypogonadotropic hypogonadism. AHC presents as adrenal failure in early infancy, although a wide range of phenotypic expressions have been reported. Interestingly, the MC2 promoter contains several DAX-1 sites. As expected, DAX-1 suppresses the expression of the MC2 gene when transfected in adrenocortical Y-1 cells. In adrenocortical tumors there is a distinct negative correlation between DAX-1 and MC2 (51-52).

Steroidogenic acute regulatory protein (StAR) does not appear to affect the MC2 promoter but regulates steroidogenesis, an effect augmented by ACTH via the MC2R. StAR promotes intra-mitochondrial cholesterol transfer in the adrenal cortical cells. StAR is thus the only major adrenal transcription factor which has not been associated with the expression of the *MC2R* gene (53).

The activator protein-1 regulatory element (AP-1) is the product of the hetero-dimerization of the proto-oncogenes *Fos* and *Jun* following activation of several signalling pathways including that of PKA and PKC. Two AP-1 binding sites have been identified upstream of the MC2R. Deletion of the AP-1 binding sites on MC2 gene abolishes the stimulatory effect of cAMP. The effect of glucocorticoids and Angiotensin II on the expression of *MC2R* gene is carried out via a glucocorticoid-mediated inhibition of AP-1 binding sites on the ACTH receptor promoter. The angiotensin II protein stimulates the expression of *MC2R* gene in the adrenal cortex. Promoter deletion studies revealed that the two AP1 binding elements on MC2 promoter mediate the Angiotensin II stimulatory signals. Indeed, Angiotensin II rapidly activates Fos and Jun to promote MC2 transcription.

The MC2 Accessory Proteins MRAP and MRAP2

For many years' researchers, in the field of adrenal physiology, suspected that an unidentified adrenal factor was needed in order for the effect of ACTH to take place. Indeed, ACTH was effective only in transfected cells with the MC2R of the adrenal lineage. In other transfected cell with the MC2R, ACTH was ineffective i.e. a crucial factor present only in cells of adrenal lineage was necessary for the effect of ACTH to take place. It was ssubsequently found that MC2R depended, for its trafficking to cell surface, on a small single trans-membrane domain protein the malfunction of which caused a clinical syndrome indistinguishable from that caused by the absence or malfunction of the MC2R. This was shown to be the MC2 accessory protein (MRAP).

MRAP is peculiar in that it naturally exists as an antiparallel homodimer formation (MRAPalpha and MRAPbeta) each pair associated with the MC2R. Later it was also shown that the MRAP protein is necessary not only for the trafficking of the receptor to cell surface, but also for conformational changes necessary for the binding of the ACTH ligand either by influencing ACTH ligand binding or by facilitating the interaction of the Gas protein with the receptor, or both (54). The MPAR gene is mapped in human chromosome 21 (C21orf61) corresponding to a murine adipocyte transmembrane protein. Two isoforms have been identified each conferring a different affinity of the MC2 receptor towards ACTH, thus explaining the observed two subpopulations of MC2 receptor as far as its affinity towards the ACTH is concerned (see above). MRAP has no effect on the trafficking of either MC1R or MC3R, while it may suppress the trafficking of MC4R and MC5R to cell surface (55-60) (Figure 4).

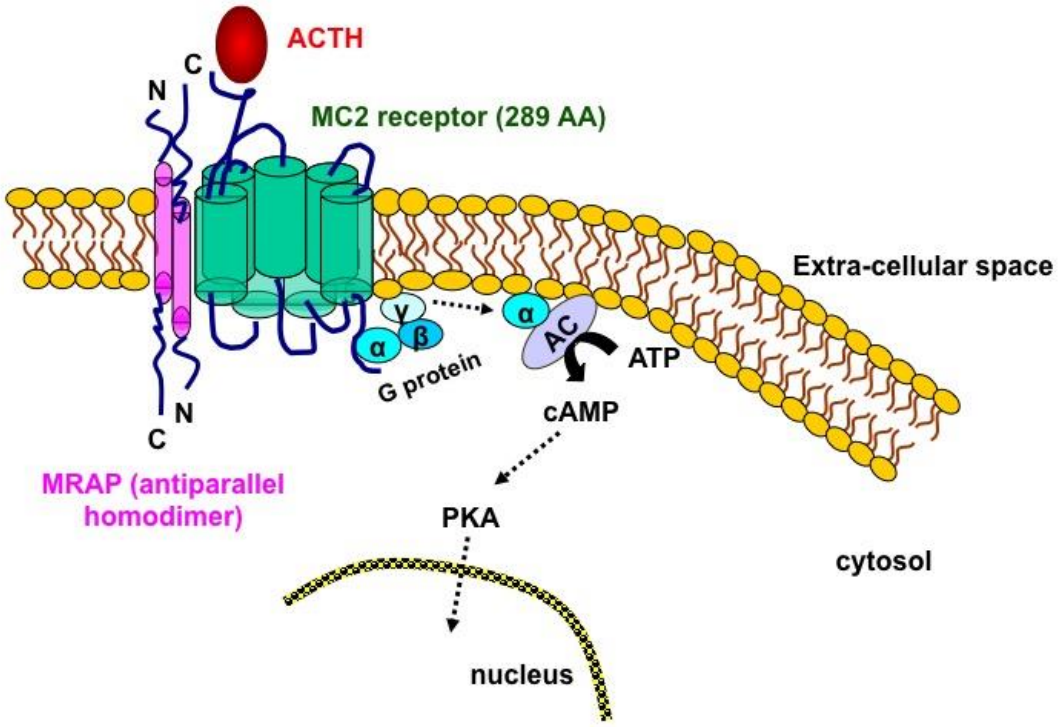

Figure 4. ACTH receptor protein expression. MC2R mRNA is translated at the endoplasmic reticulum and is unable to traffic beyond this point to the plasma membrane. MRAP mRNA is translated and adopts an anti-parallel homodimeric conformation at the endoplasmic reticulum. Only the MRAP-MC2R membrane complex is competent to bind ACTH at physiological concentrations and to generate a steroidogenic signal.

THE FAMILIAL GLUCOCORTICOID DEFICIENCY (FGD) SYNDROMES

Hereditary ACTH resistance syndromes encompass the genetically heterogeneous isolated or Familial Glucocorticoid Deficiency (FGD) and the distinct clinical entity known as Triple A syndrome. The molecular basis of adrenal resistance to ACTH includes defects in ligand binding, MC2R/MRAP receptor trafficking, cellular redox balance, cholesterol synthesis, and sphingolipid metabolism. Biochemically, this is manifested by ACTH excess in the setting of hypocortisolemia.

FGD is an autosomal recessive condition characterized by the presence of isolated glucocorticoid deficiency, classically in the setting of preserved mineralocorticoid secretion. Primarily there are three established subtypes of the disease: FGD 1, FGD2 and FGD3 corresponding to mutations in the MRC2R (25%), Melanocortin 2 receptor accessory protein MRAP (20%), and Steroidogenic acute regulatory protein STAR (5–10%), respectively. Mutations in these 3 genes account for approximately half of cases. Whole exome sequencing in patients negative for MC2R, MRAP and STAR mutations, identified mutations in mini-chromosome maintenance (MCM), nicotinamide nucleotide transhydrogenase (NNT), thioredoxin reductase 2 (TXNRD2), cytochrome p450scc (CYP11A1), and sphingosine 1-phosphate lyase (SGPL1), accounting for a further 10% of FGD. These novel genes have

linked replicative and oxidative stress and altered redox potential as a mechanism of adrenocortical damage. However, a genetic diagnosis is still unclear in about 40% of cases (61).

The FGD syndromes are autosomal recessive diseases characterized by atrophic zona fasciculata and zona reticularis, accompanied by low plasma cortisol levels and elevated ACTH. FGD syndromes exhibit an isolated defect in the endogenous production of cortisol without a parallel defect in the production of aldosterone. The cortisol insufficiency is usually accompanied by hyperpigmentation of the skin and of the mucous membranes due to the high levels of circulating ACTH activating the cutaneous MCR. Recurrent episodes of hypoglycemia are also present due to the lack of the counter-regulatory effect of cortisol on the hypoglycemic effects of insulin. The affected neonates present with failure to thrive, repeated episodes of hypoglycaemia, and seizures.

Several types of FGD are recognized as per the pathophysiological defect on the ACTH receptor pathway. The type 1 FGD and type 2 FGD cause 'pure' isolated glucocorticoid deficiency, however over the last 25 years it has become clear that glucocorticoid deficiency itself may occur as part of a syndrome with a much more complex clinical picture.

The defect in type 1 FGD is localized in the MC2R gene usually consisting of single point mutations. These inactivating mutations of the MC2R may result from the introduction of a stop codons within the coding region of the ACTH receptor, frameshift mutations, and mutations that cause single amino acid substitutions and structural disruption of the ACTH receptor affecting the ligand-binding domain resulting in loss of ligand-binding capability. Type 1 FGD represents approximately 25-40% of all patients with FGD.

The defect in type 2 FGD appears to be due to mutations in the MC2R accessory protein, the MRAP mentioned above. It represents around 15-20% of all cases of. At least 8 different mutations in MRAP have been identified in type 2 FGD patients. Most mutations of MRAP cluster around the first coding exon (exon 3) especially at the splice donor site. The same mutation has been found in genetically unrelated individuals suggesting that this is a true 'hot spot' area for mutation. The other common site for mis-sense mutations is in the initiator methionine. This mutation prevents translation of the full-length protein. The next in-frame methionine is at position 60 which, if translated, would result in a severely truncated protein. The adrenal histology of FGD type 2 is typical of all other cases of FGD. They are characterized by a relatively preserved glomerulosa cell layer with highly atrophic and disorganized fasciculata and reticularis cell layers.

The defect in type 3 FGD concerns the regulatory alacrima-achalasia-adrenal insufficiency neurologic defect (ALADIN) protein causing the Allgrove syndrome.

The defects in the remaining cases of FGD are attributed in problems within the MC2R signaling transduction. Mutations in the intracellular portion of the MC2R may result in the loss of its signal transduction properties. Absence of a biological response to ACTH may thus be due to impaired binding of ACTH to its receptors or inability of the bound ACTH to initiate its post-receptor effects (62-65).

THE ACHALASIA-ADDISONIANISM-ALACRIMA (TRIPLE A) OR ALLGROVE SYNDROME

The triple A syndrome is caused by mutations in the gene encoding the regulatory protein ALADIN, a product of the ADRACALIN gene. ALADIN is a WD-repeat regulatory protein, part of the nuclear pore complex. It is crucial for the development of the

peripheral and central nervous system. Mutations of ALADIN lead to a syndrome characterized by achalasia, alacrima, and addisonism (66-72). The underlining pathology of this syndrome appears to be a systemic and progressive loss of cholinergic function.

Alacrima is often manifested at birth, the patients exhibiting conjunctival irritation which if not treated leads to severe keratopathy and corneal dehydration-induced ulcerations. Alacrimia is diagnosed by Schirmer's test.

Achalasia is a neuromuscular disorder of the esophagus resulting in elevated lower esophageal sphincter pressure and lack of peristaltic waves of the esophagus, and recurrent lung infections resulting in respiratory failure.

The neurologic manifestations of the disease include motor neuron disease-like presentations, motor-sensory or autonomic neuropathy, optic atrophy, cerebellar ataxia, Parkinsonism, and mild dementia. The autonomic nervous system dysfunction may be manifested as papillary abnormalities, an abnormal reaction to histamine test, abnormal sweating, orthostatic hypotension, and disturbances of the heart rate. Cognitive deficits, pyramidal syndrome, cerebellar dysfunction, dysautonomia, neuro-ophthalmological signs and bulbar and facial symptoms also occur. The neurological features may appear at a later age.

Only half of the patients develop adrenal insufficiency accompanied by episodes of hypoglycemia which intensify the problems of cognition.

Using genetic linkage analysis, a causative locus has been identified on chromosome 12q13 coding the alacrima-achalasia-adrenal insufficiency neurologic defect (ALADIN) regulatory protein, a product of the ADRACALIN gene which is encoding the ALADIN protein of the nuclear pore complex. This protein is crucial in the development of the nervous system, especially its peripheral parts. Several mutations have been described including homozygous mutations of c.771delG (p.Arg258GlyfsX33) in exon 8 and c.1366C>T (p.Q456X) in exon 15 and a missense mutation in p.R155H.

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