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Steroidogenic cell microenvironment and adrenal function in physiological and pathophysiological conditions

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Abstract

The human adrenal cortex is a complex organ which is composed of various cell types including not only steroidogenic cells but also mesenchymal cells, immunocompetent cells and neurons. Intermingling of these diverse cell populations favors cell-to-cell communication processes involving local release of numerous bioactive signals such as biogenic amines, cytokines and neuropeptides. The resulting paracrine interactions play an important role in the regulation of adrenocortical cell functions both in physiological and pathophysiological conditions. Especially, recent evidence indicates that adrenocortical cell microenvironment is involved in the pathogenesis of adrenal disorders associated with corticosteroid excess. The paracrine factors involved in these intraadrenal regulatory mechanisms may thus represent valuable targets for future pharmacological treatments of adrenal diseases.

Development of the human adrenal cortex

The fetal human adrenal cortex derives from the adrenogonadal primordium that also gives rise to steroid-secreting cells in gonads. Development of fetal adrenal takes place in several stages (Xing et al., 2015). Foremost, the adrenal primordium is encapsulated by mesenchymal cells at 9 weeks post-conception. During the first trimester of gestation, the fetal gland is organized into two distinct zones: the inner fetal zone producing androgens, especially dehydroepiandrosterone (DHEA), and the outer definitive zone (ZD) without steroidogenic activity (Naccache et al., 2016; Narasaka et al., 2001). During the second trimester of pregnancy, a third zone appears between the two others. In this new area, named transitional zone (TZ), cells begin to secrete cortisol, the main glucocorticoid involved in the regulation of glucose metabolism and maturation of fetal organs (Johnston et al., 2018; Naccache et al., 2016). At the end of gestation, adrenocortical cells of the ZD gradually differentiate to synthesize the mineralocorticoid aldosterone which contributes to the maintenance of hydromineral homeostasis (Martinerie et al., 2015; Naccache et al., 2016).

Development of the adrenal cortex continues in postnatal period, in particular with the involution of the fetal zone after birth (Xing et al., 2015). The remaining cortex is thus organized in two layers producing aldosterone and cortisol, respectively. Later, a third inner layer, the zona reticularis (ZR), develops at 6–8 years of age (Belgorosky et al., 2008). Adrenocortical cells in ZR secrete adrenal androgens, mainly DHEA and its sulfate derivative (DHEA-S), which are responsible for the adrenarche (Belgorosky et al., 2008). At adolescence and adulthood, the adrenal cortex is thus subdivided into three separate histological and functional zones. All steroid hormones derive from cholesterol via a multienzyme pathway specific to each adrenocortical zone (Barnard et al., 2021; Bollag, 2014; Miller, 2018). The outermost layer, the zona glomerulosa (ZG) derived from ZD, is composed of cells arranged in rosettes and secreting aldosterone under the control of plasma potassium and the renin-angiotensin system (RAS). The middle layer, the zona fasciculata (ZF) derived from ZT, is organized in cord-like bundles of cells producing cortisol in response to the adrenocorticotrophic hormone (ACTH). The innermost layer ZR is formed by a network of cells that synthesize DHEA and androstenedione acting as precursors of sex hormones

Renewal of the adult adrenal cortex has been actively investigated during the recent past years (Pignatti et al., 2017; Walczak and Hammer, 2015; Xing et al.,

2015). Homeostasis of the gland is maintained by the balance between cell proliferation, differentiation of steroidogenic cells, centripetal migration of differentiated cells, and apoptosis of cells, the latter occurring at the cortical-medullary junction (Pignatti et al., 2017). Use of mouse models with gene deletion and cell-lineage tracing has shown that cell migration process is accompanied by a modification of cell phenotype due to differential expression of key enzymes responsible for mineralocorticoid and glucocorticoid synthesis, respectively (Freedman et al., 2013; Seccia et al., 2018).

Besides steroidogenic cells, the adrenal cortex contains various cell types, including adipocytes in the fat tissue surrounding the adrenal gland, stem cells in the capsule, immune cells infiltrating the parenchyma, neurons innervating the cortex, and endothelial cells of the dense network of capillaries irrigating the gland. There is growing evidence that these different cell types constitute a microenvironment influencing, through the release of bioactive substances, proliferation, differentiation, function and apoptosis of endocrine cells in physiological conditions. This microenvironment also seems to contribute to the pathophysiology of adrenal remodeling and steroid overproduction in adrenocortical hyperplasias and tumors. In the present review, we will expose the current knowledge on the impact of the cellular microenvironment in the physiological control of adrenal cortex and to examine its potential role in the pathophysiology of adrenal tumorigenesis and hypersecretion.

Stem and progenitor cells

Some previous reviews have well documented the existence of both stem cells located in the capsule and progenitor cells intermingled with clusters of aldosterone-producing cells in ZG, in particular in human and mouse adrenals (Walczak and Hammer, 2015; Pignatti et al., 2017; Boulkroun et al., 2011; Laufer et al., 2012; Lerario et al., 2017; Vidal et al., 2016). In addition, proliferative Ki67-positive cells have been evidenced in ZF from mouse, rat and human adrenals (Sasano et al., 1995; Engeland et al., 2005; Berthon et al., 2010). The proliferative and regenerative potentials of adrenal stem and progenitor cells are attested by the restoration of rat adrenal glands observed after enucleation (removal of the cortex leaving the adrenal capsule and adherent cortical cells) (Ennen et al., 2005).

By using different mouse models, it has been demonstrated that adrenal renewal is dependent on capsular mesenchymal cells which do not express the

transcription factor steroidogenic factor 1 (SF1) but produce different proteins, such as Wilms tumor protein homolog 1, GLI family zinc finger 1 (Gli1) or R-spondin 3, essential for maintaining the population of subcapsular SF1-positive progenitor cells (Walczak and Hammer, 2015; Pignatti et al., 2017; Lerario et al., 2017; Vidal et al., 2016; Huang et al., 2010). Gli1 is a transcriptional effector of the canonical Sonic hedgehog (SHH) pathway, while R-spondin 3 is a paracrine factor that stimulates the canonical WNT/ β -catenin pathway in the subcapsular steroidogenic cells (Vidal et al., 2016). Diverse actors of these two signaling pathways are known to be expressed in mouse and human adrenals. They are involved in regulation of adrenocortical cell proliferation, differentiation or secretion.

SHH signaling pathway

SHH released by SF1-positive CYP11B2-negative subcapsular progenitor cells stimulates centripetal migration and differentiation of Gli1-positive stem cells into Shh-producing cells (Laufer et al., 2012). The SHH-dependent paracrine communication pathway between stem and progenitor cells is crucial during development of fetal adrenal, as well as for maintaining continuous adult adrenal renewal and adapting cortex growth to particular conditions (Gomes et al., 2014; Huang et al., 2010). Interestingly, it has been reported that activation of the SHH pathway in mouse adrenals observed at cessation of long-term pharmacological glucocorticoid therapy (that induces adrenal insufficiency), facilitates progenitor proliferation, differentiation and repopulation of the cortex (Finco et al., 2018). The SHH pathway mediates the positive effect of ACTH on adrenal cortex expansion in response to activation of melanocortin 2 receptors (MC2R) which are positively coupled to the cAMP/Protein kinase A (PKA) signaling pathway (Menzies et al., 2017; Novoselova et al., 2018). By contrast, it has been observed that proliferation and recruitment of Gli1-positive stem cells are repressed by the androgenic hormone dihydroxytestosterone (Grabek et al., 2019). This observation provides a molecular basis for the sexual dimorphism of the adrenal renewal which is 3-fold more active in female than in male mice (Grabek et al., 2019), and might in part explain the high incidence of cortisol-secreting tumors in women (Stratakis, 2008). Indeed, an upregulation of the SHH signaling pathway was reported in adrenocortical adenomas and carcinomas (Boulkroun et al., 2011; Gomes et al., 2014; Werminghaus et al., 2014). Thereby, the SHH pathway might be regarded as a molecular target to lower cell proliferation in adrenal cancer.

WNT/β-catenin signaling pathway

The WNT/β-catenin pathway has been shown to be essential for adrenal fetal development and adult renewal (Basham et al., 2019; Kim et al., 2008). In subcapsular steroidogenic cells, activation of this pathway occurs in response to the release of WNT factors by progenitor cells, inducing *in fine* inhibition of β-catenin degradation (Drelon et al., 2016b). The resulting stabilization of β-catenin in the cytoplasm and its translocation into nucleus can be evidenced by immunohistochemistry in mouse and human ZG cells (Berthon et al., 2010; Boulkroun et al., 2011). At the molecular level, nuclear β-catenin acts as a transcription factor upregulating numerous target genes which determine the ZG fate. Especially, it has been recently revealed that activation of the β-catenin pathway facilitates organization of ZG cells into rosettes by inducing formation of adherens junctions, and expression of *CYP11B2*, the gene encoding aldosterone synthase, the key enzyme for aldosterone production (Berthon et al., 2010; Leng et al., 2020). Coherently, activation of the WNT/β-catenin signaling is associated with aldosterone overproduction in mouse adrenals and human aldosterone-producing adenomas (Berthon et al., 2014, 2010; Boulkroun et al., 2011). Moreover, activating mutations of *Ctnnb1*, encoding β-catenin, in mouse adrenal cortex has been linked to adrenocortical cell hyperplasia (Berthon et al., 2010; Heaton et al., 2012). By examining different mouse models with somatic activating β-catenin mutations, it has been demonstrated that hyperplastic expansion of adrenals is due to an increase in progenitor cell proliferation and/or decrease in ZG cell transdifferentiation into ZF phenotype, leading to ZG hyperplasia (Berthon et al., 2010; Pignatti et al., 2020). On the contrary, it has been shown that downregulation of β-catenin in human H295 adrenocortical cells reduces *in vivo* tumor growth in a mouse xenograft model by increasing cell apoptosis (Salomon et al., 2015). Consistent with all these observations, human genetic analyses have revealed that inactivating germline *WNT4* mutation provokes adrenal dysgenesis (Mandel et al., 2008), whereas *CTNNB1* activating mutations or inactivating mutations in genes encoding negative regulators of β-catenin, such as *APC* (adenomatous polyposis coli) and *ZNRF3*

(encoding E3 ubiquitin ligase), are frequently associated with human adrenocortical tumors (Tissier et al., 2005; Wakatsuki et al., 1998; Bonnet et al., 2011; Maharjan et al., 2018; Assié, 2018).

Recent studies have shown the occurrence of reciprocal antagonistic actions of the WNT/ β -catenin and cAMP/PKA signaling pathways on differentiation of ZG and ZF cells. Indeed, activation of the WNT/ β -catenin pathway in ZG cells represses expression of genes characteristic of ZF fate, such as *Cyp11b1* encoding 11 β -hydroxylase essential for glucocorticoid synthesis, but upregulates *Pde2a*, encoding phosphodiesterase 2a which reduces ACTH-induced cAMP production (Pignatti et al., 2020; Walczak et al., 2014). In contrast, activation of the cAMP/PKA pathway in ZF cells evoked by ACTH, downregulates *Wnt4* and inhibits activation of β -catenin, reducing the ZG phenotype (Drelon et al., 2016b). In this regard, it is noteworthy that adrenals removed from patients with 21-hydroxylase deficiency, who have chronically high plasma ACTH levels, present with alteration of the ZG histological structure. In fact, in this condition, ZG cells are not organized in rosettes but in hairpin-shaped cords, suggesting that reinforcement of the PKA signaling pathway triggered by ACTH, inhibits formation of adherens junctions which are required for rosette development (Le Mestre et al., 2019). It is now accepted that trans-differentiation of ZG to ZF phenotype is favored by a decrease in *Wnt4* concentration in the microenvironment of mouse ZG cells migrating into the deeper cortex, that results in the decline of the β -catenin pathway, and consequently emergence of the ACTH/PKA pathway. Moreover, Mathieu et al have demonstrated that the histone methyltransferase *EZH2*, which is expressed by mouse adrenocortical cells, is essential for the unidirectional transdifferentiation from ZG to ZF phenotype, and programming PKA responsiveness and priming ZF cells to respond to ACTH during adrenal cortex development and renewal (Mathieu et al., 2018). Indeed, adrenal invalidation of *Ezh2* in mice leads to ZG expansion and ZF cell hypoplasia associated with glucocorticoid insufficiency (Mathieu et al., 2018). Thus, this histone methyltransferase is indirectly involved in control of adrenocortical cell renewal. From a pathophysiological point of view, it has been recently reported that *EZH2* is the most deregulated gene in human adrenocortical carcinoma and its overexpression is associated with poor prognosis (Drelon et al., 2016a). Interestingly, blockade of *EZH2* in the H295R cell line, reduces cell proliferation and activates

apoptosis (Drelon et al., 2016a), suggesting that EZH2 may constitute a therapeutic target for the treatment of adrenal cortex cancer.

Extracellular matrix

It is now considered that extracellular matrix (EM) is an important constituent of adrenocortical microenvironment, which seems to play a significant role in renewal and maintenance of the three zones of the adrenal cortex (Lerario et al., 2017). EM is composed of proteins, such as laminin, collagens and fibronectin, and polysaccharides forming a dense network that shapes the tissues (Hynes and Naba, 2012; Lerario et al., 2017). ECM also functions as a pool of a wide variety of bioactive signals like growth factors and enzymes which, after release, can act on resident cells. The roles of ECM in the adrenal cortex have been sparsely investigated. The ECM-derived proteins fibronectin, laminin and collagen IV were shown to affect cell growth and secretory activity of human fetal adrenal cells in primary culture (Allen et al., 2010; Chamoux et al., 2002, 2001). In this model, fibronectin and laminin inhibit while collagen IV enhances ACTH-evoked cortisol synthesis. Conversely, the three proteins activate androgen synthesis. These actions result from modulation of expression of the steroidogenic enzymes 3β -hydroxysteroid dehydrogenase and 17α -hydroxylase. On the other hand, laminin and collagen IV increase cell proliferation whereas fibronectin activates cell death. It seems thus that ECM influences the responsiveness of adrenocortical cells to regulatory hormones and growth factors. Interestingly, the adrenocortical cell/ECM interaction may play a role in the pathophysiology of adrenocortical tumors. For instance, the gene encoding the matrix protein nephronectin is highly expressed in a subset of aldosterone-producing adenomas (APA) and has been shown to modulate aldosterone secretion (Teo et al., 2017). In addition, a recent study has demonstrated downregulation of several genes encoding ECM proteins in adrenocortical carcinomas in comparison with adenomas (Volante et al., 2021). Inversely, the laminin $\alpha 3$ gene was found to be overexpressed in malignant tumors. Collectively, these results suggest that proteins of ECM may represent valuable diagnostic/prognostic factors and therapeutic targets for the clinical management of adrenocortical tumors.

Immune cells

Different types of immune cells, including macrophages, lymphocytes and mast cells, are able to infiltrate the human adrenal cortex in perivascular areas, next to steroidogenic cells (Hayashi et al., 1989; González-Hernández et al., 1994; Duparc et al., 2015; González-Hernández et al., 1996). The actions of immune cells in the regulation of adrenal function have been previously described in several reviews (Marx et al., 2000; Bornstein et al., 2004; Kanczkowski et al., 2013, 2016, 2017). In particular, it has been reported that immune cells are involved in control of adrenal turnover by releasing growth factors for cell proliferation and eliminating apoptotic cells. Immune cells are also known to regulate steroid secretion, in particular in sepsis conditions (Fudulu et al., 2021; Kanczkowski et al., 2016). Indeed, the adrenal cortex can be massively infiltrated by circulating immune cells which secrete chemokines and proinflammatory cytokines, such as tumor necrosis factor- α , interleukins 1 and 6. These immune signals affect glucocorticoid production to modulate the response of the organism to infectious stress (Barney et al., 2000; González-Hernández et al., 1996; Willenberg et al., 2002). More recent studies have well documented the actions of mast cells, macrophages and lymphocytes in the human adrenal cortex in physiological and/or pathological conditions.

Mast cells in the control of aldosterone production

Mast cells derive from hematopoietic stem cells. Their progenitors circulate in the blood and migrate into peripheral tissues under the influence of the chemotactic and growth factor Kit ligand (or stem cell factor; SCF). Tissue-resident progenitors differentiate to give rise to mature mast cells. Mast cells then acquire the capacity to release various biologically active mediators, including histamine, heparin, serotonin (5-HT), cytokines, neurohormones and proteolytic enzymes. It is well established that the phenotype of mast cells infiltrating the diverse organs is determined by the host tissue environment. In particular, it has been reported that WNT factors, through activation of the β -catenin pathway, promote maturation of progenitor mast cells and increase their ability to produce cytokines and the mast cell-specific proteases chymase and tryptase (Tebroke et al., 2019; Yamaguchi et al., 2016).

Our team has extensively studied the occurrence of mast cells in human adrenals. We have observed that in fetal adrenal, Kit ligand is produced by adrenocortical cells from 18 weeks of gestation. In addition, mast cells expressing the Kit receptor (CD117) and tryptase, are detected in the definitive zone of cortex from

18 weeks of gestation, i.e. before expression of steroidogenic enzymes (Naccache et al., 2016). Thereafter, the density of mast cells progressively increases together with expression of 3 β -hydroxysteroid dehydrogenase (3 β -HSD), an enzyme involved in both cortisol and aldosterone synthesis (Fig. 1). Aldosterone synthase appears later in the definitive zone, between 33 and 40 weeks of gestation, a period during which the mast cell density is still high (Naccache et al., 2016). The presence of mast cells in the fetal adrenal prior to the appearance of steroidogenesis suggests that these immune cells may favor differentiation of adrenal progenitors into steroidogenic cells. It will be thus relevant to investigate aldosterone production in newborn mast cell-deficient mice.

In adult human adrenals, mast cells positive for CD117 and tryptase, are localized in the ZG. In mouse adrenals, mast cells whose density varies with age, gender and strain, are also confined in the ZG (Kim et al., 1997). It can be supposed that the preferential localization of mast cells in the outer cortex is determined by the production of SCF in this region (Duparc et al., 2015; Lefebvre et al., 2001; Naccache et al., 2016). Reciprocally, the presence of mast cells close to aldosterone-producing cells suggests that the immune microenvironment may influence the appearance and maintenance of the ZG cell phenotype which is dependent on the WNT/ β -catenin signaling pathway. This assumption is supported by data showing that in different organs, an increase in mast density is associated with activation of the β -catenin pathway in host tissues (Cheng et al., 2017; He et al., 2017; Lampiasi et al., 2007). Moreover, mast cell degranulation stimulates *in vitro* expression of β -catenin by tumor cell lines. These data suggest that intraadrenal mast cells might reinforce this signaling pathway in ZG, but, to our knowledge, such regulatory process has never been investigated in the adrenal gland. Conversely, the action of mast cells on aldosterone production by ZG cells has been extensively studied by *in vitro* and *in vivo* approaches. First, factors released by human mast cell line (HMC-1 and LAD2) stimulate *CYP11B2* gene expression and aldosterone secretion by the human H295R adrenocortical cell line (Duparc et al., 2015). Secondly, degranulation of mast cells induced by compound 48-80, increases aldosterone secretion by explants of rat and human adrenals (Hinson et al., 1989; Lefebvre et al., 2001). Thirdly, low-sodium diet stimulates adrenal expression of both *Tpsb2*, encoding tryptase, and *Cyp11b2* in male C57BL/6 and female BALB/c mice, which have moderate and high densities of mast cells in ZG, respectively (Boyer et al., 2017). These observations indicate that

sodium restriction activates adrenal mast cells, that in turn stimulate aldosterone synthesis. Activation of mast cells by sodium depletion may result from an indirect effect of the RAS on these immune cells, since it has been demonstrated that angiotensin II triggers rat mast cell degranulation (Carvalho et al., 2006; Kim and Im, 2019). On the contrary, there is no evidence for a contribution of mouse mast cells in the regulation of aldosterone synthesis under normal sodium diet, because both plasma and urine aldosterone concentrations are similar in wild type (WT) and mast cell-deficient mice ($Kit^{W-sh/W-sh}$) resulting from inactive mutation of Kit receptor (Boyer et al., 2017). In addition, no adrenal dysfunction has been reported in allergic subjects receiving mast cell inhibiting drugs or patients treated with imatinib which induces mast cell depletion (Cerny-Reiterer et al., 2015) but it is noteworthy that adrenocortical secretions have not been specifically explored in these conditions. Thus, the contribution of mast cells in the regulation of aldosterone production seems to be limited in the context of sodium restriction. Interestingly, disturbance of aldosterone production occurs in response to sodium depletion in the mast cell-deficient mouse model. Indeed, $Kit^{W-sh/W-sh}$ mice under low-sodium diet show an unexpected severe hyperaldosteronism consecutive to activation of the intraadrenal RAS (Boyer et al., 2017). Induction of intraadrenal RAS may represent a compensatory mechanism aimed to reinforce aldosterone synthesis and restore sodium homeostasis in the situation of mast cell deficiency. However, this process obviously overpasses its goal since it induces mineralocorticoid overproduction. This mast cell deficient mouse strain represents an animal model of primary aldosteronism, which has the particularity to be triggered by sodium restriction.

The mast cell secretory products that stimulate aldosterone synthesis have been searched for by using *in vitro* approaches. It has been reported that degranulation of human intraadrenal mast cells provokes the release of serotonin (5-HT) which triggers aldosterone production through activation of 5-HT type 4 receptors (5-HT₄R) positively coupled to the AMPc/protein kinase A pathway and L- and T-type calcium channels (Contesse et al., 2000; Lefebvre et al., 1993, 2001). The predominant localization of 5-HT₄R in ZG is consistent with the ability of 5-HT to stimulate mineralocorticoid secretion (Bram et al., 2016; Cartier et al., 2005; Le Mestre et al., 2019; Lefebvre et al., 2015). In addition, both 5-HT and 5-HT₄ receptor agonists are able to weakly increase cortisol synthesis by cultured human adrenocortical cells (Louiset et al., 2017). The cortisol response to 5-HT apparently

results from activation of 5-HT₄R present at the surface of ZG cortisol-producing cells, characterized by the absence of aldosterone synthase and expression of 11 β -hydroxylase (Louiset et al., 2017). Consistently, clinical studies have revealed that 5-HT₄R agonists (metoclopramide, cisapride, zacopride) and 5-HT reuptake inhibitors (sertraline, escitalopram) increase plasma aldosterone concentrations but do not affect circulating cortisol levels (Lefebvre et al., 1993; Edwards et al., 1980; Pratt et al., 1981; Lefebvre et al., 1995; Ahmed et al., 2011). In addition, piboserod, a 5-HT₄R antagonist, suppressed the aldosterone response to the 5-HT₄R agonist cisapride in healthy volunteers (Bharucha et al., 2000). However, the impact of piboserod on aldosterone production in basal condition or in response to other stimuli has not been investigated in this clinical trial and the compound is no more available for clinical use. Nevertheless, we have recently employed L-lysine, which acts as a partial 5-HT₄R antagonist (Smriga and Torii, 2003), to evaluate the physiological role of 5-HT in the control of aldosterone production (Duparc et al., 2017). Food supplementation with L-lysine (5–8 g/day) did not affect the plasma aldosterone variations elicited by low sodium diet or upright position. However, L-lysine intake reduced the plasma aldosterone response to the 5-HT₄R agonist metoclopramide, indicating that the aminoacid is able to antagonize adrenal 5-HT₄ receptors (Duparc et al., 2017). The inhibitory action of L-lysine was only partial in accordance with the weak 5-HT₄R antagonistic properties of the molecule. Further examination of the impact of 5-HT released by mast cells on aldosterone secretion thus requires development of potent 5-HT₄ receptor antagonists or use of telotristat, an inhibitor of 5-HT synthesis, which is now used in clinical practice for the treatment of carcinoid syndrome (Pavel et al., 2018).

Mast cells in aldosterone-producing adenomas

There is growing evidence in the literature that tumor development is frequently associated with mast cell infiltration (Aponte-López et al., 2018; Dyduch et al., 2012; Fu et al., 2017; Liao et al., 2018; Mao et al., 2018; Strouch et al., 2010). Interestingly, tumor expansion seems to be favored by growth and angiogenic factors produced by hyperplastic mast cells (Hu et al., 2018; Jachetti et al., 2018; Mukai et al., 2018). In particular, it has been reported that mast cells exert positive actions on the growth of endocrine neoplasias, including thyroid and pancreas tumors (Ammendola et al., 2017; Visciano et al., 2015).

The role of mast cells in the pathophysiology of adrenocortical tumors has also been investigated. Adrenal mast cell infiltration was documented for the first time in few tumors producing either deoxycorticosterone or aldosterone but not in cortisol-secreting adenomas (Aiba et al., 1985). Subsequently, a transcriptomic analysis has revealed that APAs overexpress *KITLG*, encoding the KIT ligand, and numerous mast cell genes in comparison with normal adrenals (Duparc et al., 2015). In addition, mast cell hyperplasia has been found in a large series of APAs (Duparc et al., 2015). High density of mast cells can be explained by the diffuse expression of KIT ligand in APAs (Duparc et al., 2015). However, the potential role of mast cell proliferation on the activation of the WNT/ β -catenin signaling pathway observed in APAs (Berthon et al., 2014) has not been explored.

The distribution of mast cells in adrenal lesions responsible for primary aldosteronism distinguishes two APA subtypes (Duparc et al., 2015). In the major subtype, mast cells are predominant in the peritumoral cortex adjacent to adenoma. Such pattern of mast cell distribution is more frequently detected in female patients and in the largest tumors. Conversely, there was no correlation between mast cell density and plasma aldosterone levels in this subset of tumors (Duparc et al., 2015). These findings suggest that peritumoral mast cells do not influence aldosterone production but rather regulate proliferation of adenoma cells. In this respect, it has been reported that in other organs, peritumoral mast cells promote tumor expansion more efficiently than intratumoral mast cells (Marichal et al., 2013). The preferential localization of mast cells in aldosterone-producing micronodules located in the peritumoral cortex also indicates that mast cells control aldosterone biosynthesis in the tissue adjacent to the adenoma (Duparc et al., 2015). Thus, the presence of mast cells might explain the maintenance of *CYP11B2* expression in multiple aldosterone-producing micronodules (Boukroun et al., 2010).

The second subtype of APAs which is characterized by a higher mast cell density in the adenoma, has predominant expression of *CYP11B2* in tumors (Duparc et al., 2015). In these patients, the mast cell density in adenomas correlates with both plasma aldosterone and aldosterone/renin ratio levels. These observations indicate that intratumor mast cells exert a positive action on aldosterone production in this APA subtype. It is interesting to notice that the highest mast cell densities in APAs have been observed in tissues with somatic *CACNA1D* mutations responsible for an increase in calcium signaling and aldosterone overproduction. It seems conceivable

that elevation of calcium concentration in *CACNA1D*-mutated steroidogenic cells might stimulate production of KIT ligand enhancing mast cell chemoattraction in adenomas. Indeed, it has been reported that treatment of mice with nifedipine, a L-type calcium channel blocker, reduces expression of KIT ligand and mast cell infiltration in cardiac tissues of a mouse model of myocarditis (Liu et al., 2009).

Collectively, these findings suggest that, depending on their preferential peri- or intra-adenomatous localization, mast cells may contribute to the pathogenesis of APA through a proliferative or steroidogenic effect, respectively. It can also be hypothesized that aldosterone excess may in turn influence the activity of mast cells. Indeed, it has been reported that in the mouse cardiovascular system, mast cells express the mineralocorticoid receptor and eplerenone, a selective aldosterone receptor antagonist, reduces production of different mast cell proteases in heart (Schjerning et al., 2013; Xiao et al., 2009). Globally, aldosterone overproduction in APAs may favor mast cell hyperplasia leading to the development of a positive amplification loop reinforcing mineralocorticoid synthesis.

Macrophages

Infiltration of the adrenocortical tissue by macrophages has been noticed in human, rat and mouse adrenal glands (González-Hernández et al., 1994; Almeida et al., 2004; Ishii et al., 2012). Macrophages with ultrastructural characteristics of lysosomal activity, are especially abundant in the ZF and in ZR, the latter a region with high cell apoptosis (González-Hernández et al., 1994; Almeida et al., 2004; Mitani et al., 1999). In rat, the density of macrophages in the inner cortex increases with age and after dexamethasone-treatment inducing cortex regression (Almeida et al., 2004). Enhancement of macrophage activity may lead to removal of steroidogenic dead cells through a phagocytosis process, and cortex atrophy in response to corticoid treatment (Almeida et al., 2004). Cortex infiltration by macrophages can also be potentiated by cytokines, such as interleukin 6, produced by adrenocortical cells during inflammation. Such pathophysiological mechanism has been illustrated in adrenals of mice deficient in the steroidogenic acute regulatory protein (Star), the protein involved in cholesterol transfer into the inner mitochondrial membrane (Ishii et al., 2012). In this mouse model of congenital lipid adrenal hyperplasia, the inflammatory response of adrenocortical cells to accumulation of cytosolic cholesterol esters is associated with massive macrophage infiltration. In turn, resident macrophages can release cytokines affecting adrenal cholesterol metabolism and

expression of steroidogenic enzymes (Bornstein et al., 2004; Ishii et al., 2012; Kanczkowski et al., 2016). These observations support the existence of bidirectional interactions between adrenocortical cells and resident macrophages to adapt steroidogenesis to particular inflammation conditions. Moreover, both inflammation and infiltration by CD68-positive macrophages have been reported in cortisol-producing adenomas (Kitawaki et al., 2018), suggesting that this immune cell type might control adrenal tumor expansion, as reported for other neoplasms (Qian and Pollard, 2010). Current studies are focusing on the role of intraadrenal macrophages in genetic modified mouse models of adrenal tumors to elucidate their functions in the pathogenesis of adrenal carcinoma and metastasis.

Lymphocytes

Numerous studies have demonstrated the presence of lymphocytes in the vicinity of adrenocortical cells in human adrenals that favors paracrine communications between the two cell types. In this respect, Bornstein and collaborators have shown that cytokines released by lymphocytes modulate adrenal steroidogenesis, notably to adapt glucocorticoid secretion to infectious stress (Marx et al., 2000). Lymphocytes stimulate androgen secretion by ZR cells (Wolkersdörfer et al., 1999). Moreover, a large population of T lymphocytes has been found associated with extensive destruction of the parenchyma in adrenals from patients with Addison's disease resulting from production of autoantibodies against the steroidogenic enzyme 21-hydroxylase (Krywaczyk and Bundock, 2017; Hellesen et al., 2018). Similarly, adrenal cortex infiltration by numerous T lymphocytes has been reported in a humanized mouse model after treatment with anticancer checkpoint inhibitors targeting CTLA-4, known to induce autoimmune related adverse events (Vudattu et al., 2014). Such T lymphocyte infiltration process allows understanding the mechanism responsible for adrenalitis in patients treated by cancer immunotherapy (Paepegaey et al., 2017; Trainer et al., 2016). Globally, these observations indicate that T lymphocytes are involved in elimination of adrenocortical cells in the context of autoimmune diseases.

Recent data suggest that T lymphocytes might also affect the survival of steroidogenic cell in adrenal tumors. This hypothesis is supported by the detection of numerous lymphocytes in both aldosterone- and cortisol-producing adenomas (CPAs) (Guadagno et al., 2020; Kitawaki et al., 2018). Immunohistological studies

have revealed that CD3/CD4-and CD3/CD8-positive T lymphocytes are predominant over B lymphocytes, and are especially abundant in CPAs (Kitawaki et al., 2018). The major presence of T-lymphocytes in CPAs over APAs was unexpected since glucocorticoids exert profound anti-inflammatory effects, whereas aldosterone is well known to trigger inflammation, in particular in the cardiovascular system. The predominant recruitment of T lymphocytes in CPAs has been explained by overexpression of genes encoding chemokines with chemoattractant actions, including CXCL1, 2, 5 and 12, in CPA tissues in comparison with APAs (Kitawaki et al., 2018). Nevertheless, the observation that expression of *CXCR4*, encoding the receptor for the chemokine CXCL12, is correlated with *CYP11B2* in APAs led two groups to use the specific CXCR4 ligand 68Ga-pentixafor to localize APAs by positron emission tomography in patients with primary aldosteronism (Ding et al., 2020; Heinze et al., 2018). Targeting CXCR4 with a radioligand for medical imaging might represent a non-invasive alternative to adrenal vein sampling for differential diagnosis between APA and bilateral adrenal hyperplasia.

It is interesting to note that in cortisol-producing lesions, the density of T lymphocytes is globally higher in adenoma than in carcinoma tissues (Guadagno et al., 2020). These data indicate that T lymphocyte density represents a potential diagnostic parameter to discriminate between benign and malign adrenocortical lesions (cutoff > 10 cells/mm²), in combination with Weiss score and Ki67 index (Guadagno et al., 2020). Additionally, the highest scores of T lymphocyte infiltration in carcinomas correlate with best overall patient survivals (Landwehr et al., 2020). By contrast, T lymphocyte depletion in adrenocortical carcinomas and metastasis is associated with poor survival especially in patients with cortisol oversecretion, indicating that glucocorticoid excess hampers T cytotoxic lymphocyte ability to eradicate tumor cells (Landwehr et al., 2020). The lack of T lymphocytes in adrenocortical carcinomas responsible for hypercortisolism explains why immunological treatments, that target the programmed cell death-1 (PD-1), have a modest efficacy in patients with severe Cushing's syndrome (Carneiro et al., 2019; Habra et al., 2019; Le Tourneau et al., 2018). Reciprocally, the presence of immune cells in adrenal neoplasms could reveal the tumor sensitivity to cancer immunotherapy. In the same way, *in silico* studies are currently investigating the value of immune-related biomarkers as potential indicators for immunotherapy (Peng et al., 2020). Interestingly, treatment with pembrolizumab, a PD-1 checkpoint

inhibitor, increased T lymphocyte infiltration and reduced tumor growth in a cancer model developed by xenograft of adrenocortical cells derived from a patient's carcinoma in humanized mice (Lang et al., 2020). Administration of pembrolizumab to the same patient subsequently reduced the size of lung and hepatic metastases. Taken together, these data suggest that the efficacy of cancer immunotherapy in patients with adrenocortical carcinomas might be enhanced by inhibiting glucocorticoid steroidogenesis first to prevent the anti-inflammatory action of cortisol on intratumoral T lymphocytes.

Adrenal infiltration by immune cells has also been observed in non-neoplastic diseases. In fact, a recent post-mortem histological study gave evidenced for adrenal infiltration by CD3⁺ and CD8⁺ T-lymphocytes in patients with coronavirus disease 2019 (COVID-19) (Zinserling et al., 2020).

Innervation

The adrenal is richly innervated by nerve fibers of both intrinsic and extrinsic origins. The intrinsic innervation of the inner cortex originates from cell bodies localized in the medulla (Charlton et al., 1991; Mravec, 2005). The extrinsic innervation includes numerous sympathetic and parasympathetic fibers originating from the splanchnic and vagus nerves. Nerve fibers reach the adrenal gland with blood vessels, form a plexus in the subcapsular region and project into the ZF (Dorovini-Zis and Zis, 1991; Ehrhart-Bornstein et al., 1998; Engeland, 1998; Li et al., 1999; Whitworth et al., 2003). Varicose axon terminals are detected close to blood vessels irrigating the gland and in the vicinity of parenchyma cells (Ehrhart-Bornstein et al., 1998; Whitworth et al., 2003).

In rat and human adrenal cortices, innervation is composed of fibers producing the catecholamine synthesizing enzyme tyrosine hydroxylase, acetylcholine, NO synthase and diverse neuropeptides including cholecystokinin, dynorphin, somatostatin, vasoactive intestinal polypeptide, calcitonin gene-related peptide and substance P (Ehrhart-Bornstein et al., 1998; Heym et al., 1995; Colombo-Benkmann et al., 1996; Heym, 1997; Tóth et al., 1997; Li et al., 1999; Murabayashi et al., 2007; Wils et al., 2020). The plasticity of adrenal innervation has been evidenced by neurite outgrowth observed in the ZG of rats fed with low sodium diet. As a matter of fact, sodium depletion provokes elongation of VIP and NPY fibers in parallel with both ZG expansion and increase in aldosterone synthase expression (Tran et al., 2010).

These observations indicate that adrenal innervation may be involved in control of steroidogenic cell proliferation, differentiation and/or secretory activity.

The stimulatory action of the nervous system on adrenal renewal has been revealed in the 2000s by using rat models of unilateral adrenalectomy, adrenal enucleation and denervation by local treatment with the neurotoxin capsaicin that reduces the CGRP and substance P content in neurons (Heym et al., 1995; Ulrich-Lai et al., 2003, 2002; Ulrich-Lai and Engeland, 2000). In particular, it has been reported that activation of capsaicin-sensitive fibers participates in the compensatory growth of the contralateral adrenal after unilateral adrenalectomy (Ulrich-Lai et al., 2003, 2002). By contrast, *in vivo* treatment with SP receptor antagonist induces atrophy of ZG in rat (Mazzocchi et al., 1995).

The role of innervation in the regulation of adrenal steroidogenesis has been previously documented in several reviews (Whitworth et al., 2003; Ehrhart-Bornstein et al., 1998, 2000; Engeland, 2013). For instance, it has been reported that in rat, activity of sympathetic neurons enhances diurnal glucocorticoid secretion by increasing the adrenocortical cell responsiveness to ACTH (Engeland, 2013; Ulrich-Lai et al., 2006), whereas diverse neuropeptides including substance P, neuropeptide Y, neurotensin and Leu-enkephalin, stimulate aldosterone production (Hinson et al., 1994; Nussdorfer et al., 1988; Nussdorfer and Malendowicz, 1998).

More recent studies have detailed the role of substance P, a neuropeptide belonging to the tachykinin family, in the human adrenal cortex (Wils et al., 2020). It has been observed that both *TAC1* and *TACR1* genes, encoding respectively substance P and its receptor, named neurokinin 1 (NK1), are expressed in human adrenal (Pinto et al., 2004; Wils et al., 2020). Substance P positive fibers are present in ZG in close contact with aldosterone-producing cells which express NK1 receptors (Heym, 1997; Wils et al., 2020) (Fig. 2). Functional studies have shown that in cultured adrenocortical cells, substance P enhances expression of *HSD3D2* and *CYP21A2*, two genes encoding steroidogenic enzymes involved in both glucocorticoid and mineralocorticoid synthesis (Wils et al., 2020). In addition, substance P is a more potent stimulator of aldosterone than of cortisol secretion. As expected, the aldosterone response to substance P is inhibited by the NK1 receptor antagonist aprepitant. Examination of the transduction mechanism associated with the adrenal NK1 receptor has revealed involvement of the ERK/MAP kinase signaling pathway, like for angiotensin receptor type 1 (AT1R) (Fig. 3). However, unlike AT1R,

NK1 receptors are weakly coupled to the phospholipase C/calcium signaling pathway in human adrenocortical cells. Although substance P has been reported to activate the WNT/ β -catenin pathway in melanocytes, osteoblasts and β -pancreatic cells (Mei et al., 2014; Zhou et al., 2016; Zhang et al., 2018), such coupling system has not yet been investigated in aldosterone-producing cells. It can be hypothesized that stimulation of ZG cells by substance P-ergic fibers might contribute to β -catenin activation in subcapsular steroidogenic cells. Globally, these data indicate that the autonomic nervous system directly stimulates aldosterone secretion through substance P-ergic neurons (Fig. 4).

Substance P may also exert indirect influences on adrenal steroidogenesis. In fact, the presence of both substance P fibers and NK1 receptors in the wall of arteries irrigating the human adrenal cortex (Wils et al., 2020), suggests that the neuropeptide might regulate mineralocorticoid synthesis by increasing blood flow, as previously reported in rats (Hinson et al., 1994) (Fig. 4). We can also consider a putative indirect effect of substance P *via* activation of intraadrenal mast cells. Actually, it has been reported that, like in other organs, adrenal mast cells are innervated by subcapsular fibres (Duparc et al., 2015; Levick et al., 2019; Wang et al., 2014). Moreover, in many tissues, mast cell degranulation is triggered by nerve fibres through release of SP (Levick et al., 2019; Wang et al., 2014). It would thus be interesting to search for the presence of substance P-positive fibers in close contact to mast cells in the human adrenal cortex. The respective roles of substance P resulting from its direct action on steroidogenic cells and indirect effects mediated by vasodilation or mast cell degranulation could be explored by using different strains of cell-specific NK1 receptor knock-out mice.

The physiological role of SP in the regulation of human aldosterone production has been investigated *in vivo* by using aprepitant, a NK1 receptor antagonist commonly prescribed as an anti-emetic drug for the management of chemotherapy-induced nausea and vomiting (Hesketh et al., 2017). A placebo-controlled double-blind study was conducted in 20 healthy volunteers and revealed that aprepitant (125 mg for one day and 80 mg for 3 days orally) reduces global aldosterone production (-28% on daily urinary aldosterone excretion) (Wils et al., 2020). Interestingly, aprepitant lowers plasma aldosterone level in subjects in recumbency but does not affect their circulating concentration of aldosterone in upright position. The inhibitory

effect of aprepitant on aldosterone level in recumbency is independent of any plasma renin, potassium and ACTH fluctuations. These observations reveal that adrenal innervation by substance P-ergic fibers exerts a stimulating tone on steroidogenic cells to maintain mineralocorticoid production in supine position. This new mechanism of regulation appears complementary to the renin-angiotensin system which is activated in situations requiring strong elevation of aldosterone production, like adaptation to upright position and sodium depletion.

Up to date, no defect of substance P-ergic innervation has been associated with aldosterone dysregulation. However, it can be hypothesized that overactivation of the autonomic nervous system, as observed in patients with obesity and sleep apnea syndrome, might be responsible for aldosterone overproduction which is largely independent of the renin angiotensin system in these two conditions (da Silva et al., 2009; Dudenbostel and Calhoun, 2012; Bisogni et al., 2016; Ohno et al., 2018). Conversely, it is conceivable that a loss of the SPergic control of aldosterone secretion may be involved in the pathogenesis of hypoaldosteronism observed in diabetic patients with autonomic neuropathy (Sousa et al., 2016).

These observations also offer interesting therapeutic perspectives. Especially, NK1R antagonists like aprepitant may represent valuable pharmacological tools to reduce obesity- and SAS-associated hyperaldosteronism. Counteracting the adrenal SPergic command with these drugs may also reduce the progression of heart failure and chronic nephropathy as it is now well demonstrated that aldosterone exerts deleterious effects in the cardiovascular system and kidney (Briet and Schiffrin, 2010; Gorini et al., 2018).

In conclusion, adrenocortical cells are markedly influenced by their microenvironment both in physiological and pathophysiological conditions. The cell types which interact with adrenal steroidogenic cells especially include mast cells, macrophages, lymphocytes and neurons through local release of various bioactive signals. These cell-to-cell communication processes are involved in the pathogenesis of adrenal disorders and represent thus relevant targets for future pharmacological treatments of steroid excess syndromes.

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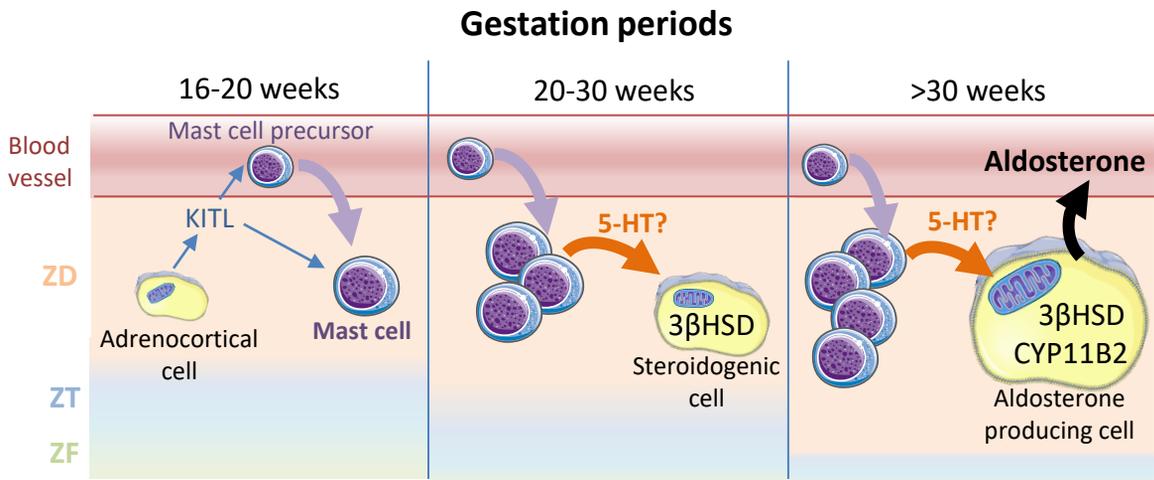
Fig. 1. The paracrine mast cell/adrenocortical cell interaction in the developing human fetal adrenal gland. From 16 weeks of gestation, adrenocortical cells produce the mast cell growth factor KIT ligand (KITL) that act as a chemoattractant agent on circulating mast cell precursors. Under its influence, mast cell precursors migrate into the definitive zone (ZD) of the fetal adrenal cortex and progressively differentiate into mature mast cells. The increase in mast cell adrenal density is then associated with expression of steroidogenic enzymes suggesting that mast cell secretory products, like serotonin (5-HT), promote the establishment of the mineralocorticoid synthesizing pathway. Conversely, mast cells are not detected in the transitional zone (ZT) and fetal zone (ZF). 3 β HSD, 3 β hydroxysteroid dehydrogenase; CYP11B2, aldosterone synthase.

Fig. 2. Substance P-ergic innervation of the human adrenal gland. Immunohistochemical studies showing the presence of substance P (SP) fibres around clusters of steroidogenic cells (A) and neurokinin type 1 (NK1) receptor in adrenocortical cells (B) in the zona glomerulosa (ZG) of the adrenal cortex. Immunohistological experiments were performed with antibodies against SP (clone NC1/34; Interchim) and NK1 receptor (#T5950; Sigma-Aldrich). For details of the Methods, please see (Wils et al., 2020).

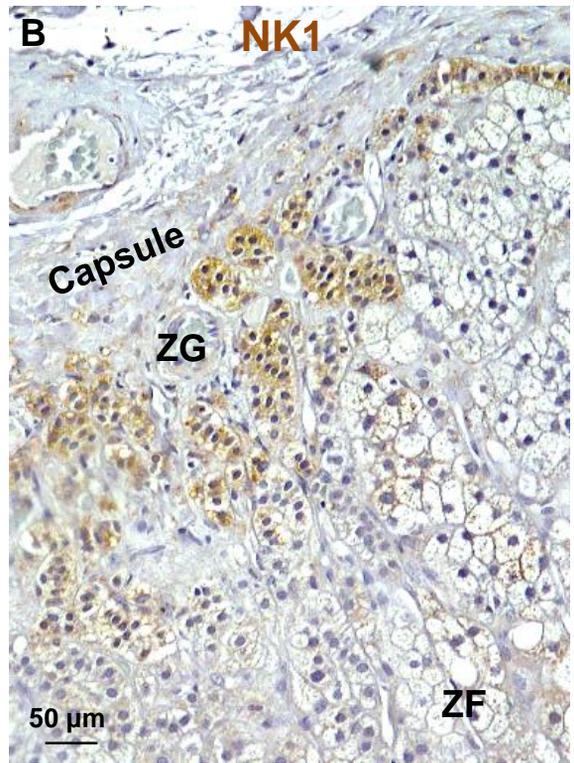
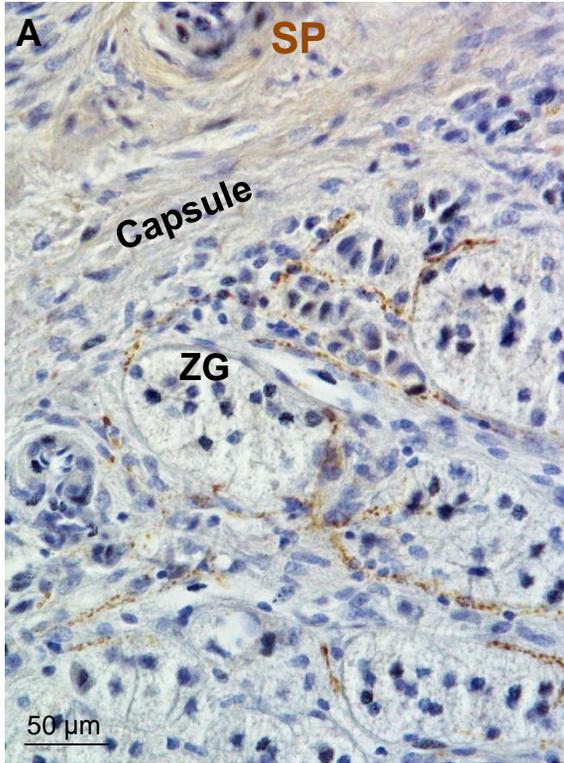
Fig. 3. Comparison of the action mechanisms of substance P and angiotensin II in human adrenocortical cells. Schematic representation of the transduction mechanisms of the NK1 and angiotensin type 1 (AT1) receptors in aldosterone-producing cells. Binding of SP to the NK1 receptor activates the ERK1/2 pathway but has a weak effect on the calcium signaling pathway, in comparison with Ang II which strongly stimulates the two transduction mechanisms.

Fig. 4. Schematic representation of the different actions of substance P involved in the control of aldosterone production in the human adrenal gland. Substance P-ergic (SP) neurons exert a direct stimulatory effect on aldosterone secretion *via* activation of neurokinin 1 receptor (NK1R) expressed by adrenocortical aldosterone-producing cells (left). Substance P might also increase aldosterone secretion through indirect effects. First, SP may activate intraadrenal mast cells which release serotonin (5-HT)

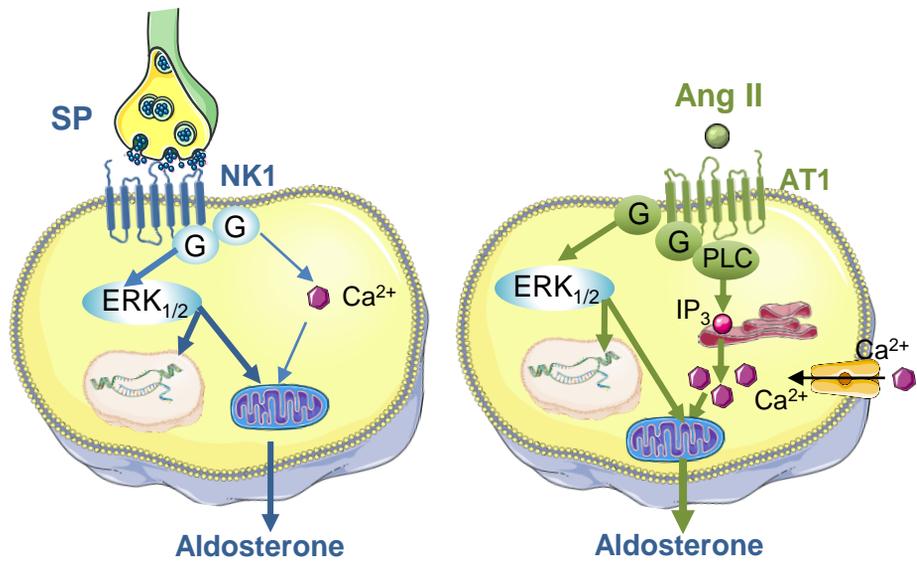
in the vicinity of adrenocortical cells expressing the 5-HT₄ receptor (middle). Second, SP may exert its vasorelaxant action on adrenal arteries leading to an increase in blood flow and activation of steroidogenesis (right).



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Fig. 1

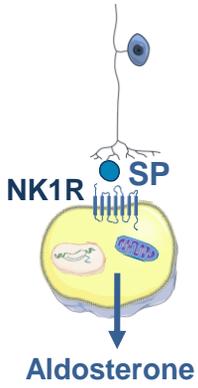


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Fig. 2

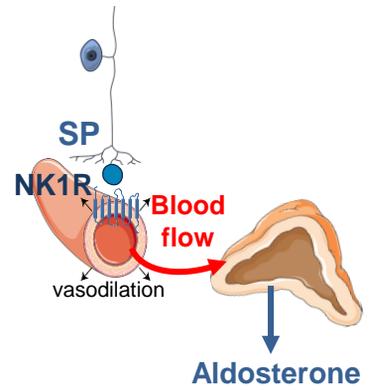
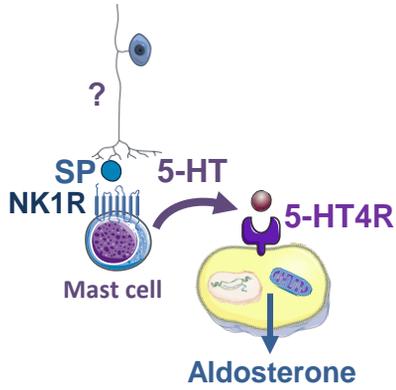


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Fig. 3

Direct effect



Indirect effects



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Fig. 4