Advances in steroid research from the pioneering neurosteroid concept to metabolomics: New insights into pregnenolone function

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Highlights:

- New neuromodulators and production sites have been discovered in steroid research
- Neurosteroids are brain messengers that are produced in the nervous system
- Rapid neurosteroid action involves membrane ionotropic and G-coupled receptors
- Pregnenolone binds to and is a novel signaling specific inhibitor of the GPCR, CB1R
- Metabolomics is a promising tools for addressing neurosteroid-related disorders

Keywords: Neuroendocrinology; Homeostasis; Sites of steroid production; Neuromodulation; Neurosteroids; Pregnenolone; Membrane receptors; CB1R; Mass spectrometry; Metabolomics

ABSTRACT

Advances in neuroendocrinology have led to major discoveries since the 19th century, identifying adaptive loops for maintaining homeostasis. One of the most remarkable discoveries was the concept of neurosteroids, according to which the brain is not only a target but also a source of steroid production. The identification of new membrane steroid targets now underpins the neuromodulatory effects of neurosteroids such as pregnenolone, which is involved in functions mediated by the GPCR CB1 receptor. Structural analysis of steroids is a key feature of their interactions with the phospholipid membrane, receptors and resulting activity. Therefore, mass spectrometry-based methods have been developed to elucidate the metabolic pathways of steroids, the ultimate approach being metabolomics, which allows the identification of a large number of metabolites in a single sample. This approach should enable us to make progress in understanding the role of neurosteroids in the functioning of physiological and pathological processes.

1. Introduction

Steroid-brain interactions are involved in a wide range of neurofunctional processes, and the consideration of regulatory loops has led to a better understanding of the action of hormone steroids on adaptive processes. The study of these interactions has become one of the most innovative chapters in modern neuroendocrinology. Classically, these interactions have been attributed to steroids synthesized in the periphery and acting in the brain. However, there is now evidence that some steroids can be neosynthesized in the brain (in neurons and glial cells) independently of peripheral sources. This discovery led to the concept of "neurosteroids". Thus, the nervous system is the target of two types of steroids, distinguished by the site of production: steroids synthesized by peripheral tissues and neurosteroids synthesized directly by nerve cells. In parallel with this discovery, the term "neuroactive steroids" was proposed to describe the relatively rapid neuromodulatory action of steroids on

membrane targets. Since the concept of neurosteroids was postulated, numerous studies have sought to understand the functional role of neurosteroids by investigating their synthetic pathways, targets of action, and neuromodulatory effects. In addition, considerable effort has been devoted in recent years to the development and validation of neurosteroid assays in the nervous system, with particular emphasis on measuring changes in endogenous neurosteroids under physiological and pathological conditions. Thus, metabolomic approaches, including lipidomics and steroidomics, are increasingly being studied to identify possible imbalances in lipid and steroid metabolism in relation to specific pathologies.

These approaches revealed a new role for neurosteroids, and in particular for pregnenolone, long thought to be an inactive precursor of neurosteroids.

2. Current dogmas in neuroendocrinology: update on sites of steroid production and molecular targets

2.1. Brief history of advances in neuroendocrinology

The history of the field of neuroendocrinology has been described in several reviews (Coen, 2015; Fink, 2015; Kreier and Swaab, 2010; Meites, 1992; Modlin et al., 2006; Plant, 2015; Tsutsui, 2016; Watts, 2015a). Table 1 provides a brief overview of the major advances in neuroendocrine research. The recognition of the brain as a hormone-secreting gland is tied to the emergence of the concept of homeostasis, first described by Claude Bernard (1813–1878) and then expanded and formulated by Walter Bradford Cannon (1871-1945) (Cannon, 1939). This concept refers to the state of maintaining an internal environment (“milieu intérieur”) close to constancy in the face of external perturbations, as well as to the implemented self-regulation process (Cooper, 2008; Libretti and Puckett, 2023). More recently, homeostasis has been described as a dynamic disequilibrium (Billman, 2020). The scope of neuroendocrinology then includes neuroendocrine adaptations to maintain or restore

homeostasis through neuroendocrine responses to external challenges, as well as neuroendocrine feedback and feedforward regulation.

Neuroendocrinology initially focused on the control of pituitary hormone secretion by the hypothalamus and then on the study of bidirectional interactions between neurons and endocrine glands. The history of neuroendocrinology begins in the 2nd century with the theory of Claudius Galenus (131-216 AD), which was developed in the 16th century by Andreas Vesalius (1514-1564), who postulated that the brain excreted a waste residue (called *pituita*) from animal spirits that was distributed throughout the body by the nerves (Oakes et al., 2016a, 2016b). At the same time, the hypothalamus-pituitary region has been a source of inspiration for a number of Renaissance artists, including Leonardo da Vinci (1508-1509) and Michelangelo Buonarroti (1475-1564), who used the brain diagram (including hypothalamus, pituitary and brainstem) as the background for his painting 'The Creation of Adam' on the ceiling of the Sistine Chapel in the Vatican (Rome, Italy) (Lechan and Toni, 2000).

The mid-1950s was a period of debate about how the brain controlled the pituitary. Neuroendocrinology as a neuroscientific perspective emerged with the spatial representation of function in the brain, including neuroendocrine control of the hypothalamic-pituitary-gonadal axis (Harris, 1955). Harris's work was at the origin of the discipline of neuroendocrinology as he stated that “it is clear that the interplay between the central nervous system and the endocrine glands... is one of reciprocity” . His work was recognized with the award of the Dale Medal by the Society for Endocrinology in 1971 (Harris, 1972). During the same period, the journal *Neuroendocrinology* was founded in 1967 and the Nobel Prize in Medicine was awarded in 1977 to Andrzej Wiktor Shally and Roger Guillemin for their discoveries of peptide hormone production in the brain (hypothalamic peptide releasing factors) (Guillemin and Rosenberg, 1955; Saffran et al., 1955). These neuropeptides are conserved across species and are essential for physiological functions. Then, following the

work of pioneers Hans Selye (1907-1982) and Claude Fortier (1921-1986), the hypothalamic-pituitary-adrenal (HPA) axis was described (Jackson, 2014; Rosténe, 2005; Selye, 1946; Vale et al., 1981). Later, technical advances helped to determine the organization and localization of hypothalamic neuroendocrine neurons, as well as the content of neuropeptides and neurotransmitters (Everitt et al., 1986; Hökfelt et al., 1986; Swanson, 1983).

Women were also pioneers in the establishment of neuroendocrinology (Breathnach and Moynihan, 2013). The recognition of women in science came after the Nobel Prizes in physics in 1903 and in chemistry in 1911, awarded to Marie Sklodowska Curie (1867-1934) and to Irene Joliot-Curie (1897-1956) in chemistry in 1935. In the field of neuroendocrinology, the concept of control theory in homeostatic mechanisms was strengthened by the work of women scientists such as Mary Pickford (1902-2002) and Berta Scharrer (1906-1995), with the discovery that the posterior pituitary gland was not only a gland, but the site of release of vasopressin and oxytocin into the circulation from fibers in the hypothalamic-hypophysial tract (Pickford, 1975; Scharrer, 1975). In addition, the major discoveries of Dora Jacobsohn (1908-1983), Dorothy Price (1899-1980) and Martha Vogt (1903-2003) include the regulation of the anterior pituitary, the feedback of steroid hormones to the pituitary (Jacobson, 1975; Price, 1975; Vogt, 1975). In 1977, the Nobel Prize was awarded to Rosalyn Yalow (1921-2011) for the development of radioimmunoassays for the measurement of small biological molecules in fluids (Yalow, 1973).

Further advances in neuroendocrinology were made with the characterization and brain localization of G protein-coupled receptors (GPCRs) involved in the endocrine action of hypothalamic releasing factors to mediate neuroendocrine responses to environmental and physiological challenges (van den Burg and Neumann, 2011). Then, in 2012, the Nobel Prize in Chemistry was awarded to Brian Kobilka (1955-) and Robert Lefkowitz (1943-) for their work on understanding the structure/function of GPCRs, which formed the basis of GPCR

molecular endocrinology, unravelling the mechanisms of hormone and neurotransmitter recognition (Bockaert, 2012; Hausch and Holsboer, 2012). In addition to understanding the mechanisms involved in the regulation of the endocrine system and peptide synthesis, neuroendocrinology has evolved into the clinical diagnosis and treatment of neuroendocrine-related diseases (Fink, 2015).

The field of neuroendocrinology has been, and still is, based on several axes involving relationships between the brain and the production of peripheral steroid hormones through feedback loops. The emergence of the concept of neurosteroids, based on the production of steroids "by the brain for the brain", is a major advance in the history of neuroendocrinology.

2.2. Sites of steroidogenesis: including the brain

2.2.1. Steroid production from cholesterol

In all steroidogenic tissues, including the brain, the initial steps of *de novo* steroid biosynthesis involve the mobilization of cholesterol into the outer and then inner mitochondrial membranes (OMM and IMM, respectively) (Poderoso et al., 2009) and its subsequent cleavage to pregnenolone by the cytochrome P450 side chain cleavage enzyme, P450scc (encoded by the gene Cyp11a1) (Papadopoulos and Miller, 2012; Payne and Hales, 2004). This first step in the conversion of cholesterol to pregnenolone is conserved across species - present from amphibians to mammals - and is common to all sites of steroidogenesis, where it is a prerequisite for the design of a new source of steroid production (Bassi et al., 2021; Payne and Hales, 2004) (see graphical abstract).

The production of pregnenolone from cholesterol involves several stages, including the availability of free cholesterol, its transport across mitochondrial membranes to the mitochondrial matrix and its subsequent cleavage to pregnenolone (see Figure 1 for steroidogenic brain cells). Cholesterol is an essential and tightly regulated component of membranes, maintaining membrane fluidity. The excess cholesterol of membrane lipids,

referred to as active or free cholesterol, constitutes the pool of available substrate for steroidogenesis after its uptake into intracellular membranes (Miller and Bose, 2011). General steroid biosynthesis is derived from approximately 2% of the daily cholesterol pool in humans, derived from dietary sources and *de novo* synthesis from acetyl-CoA. To initiate and maintain steroidogenesis, a constant supply of cholesterol must be available within the cell and delivered to mitochondrial membranes, which have a relatively low cholesterol content (Kraemer et al., 2017). There are several sources of cholesterol for steroidogenesis. The best-characterized sources of cholesterol in steroidogenic cells have been described in the adrenals and gonads. First, cholesterol can be *de novo* synthesized from acetate in the endoplasmic reticulum (ER) and transported from the ER to the plasma membrane and then to the OMM. Secondly, cholesterol contained in lipoprotein-derived cholesteryl esters (low and high-density lipoproteins, LDL and HDL, respectively) can be delivered to cells. Free cholesterol can be produced by the endocytic pathway within lysosomes or by the hydrolysis of cholesteryl esters stored in lipid droplets via the hormone-sensitive lipase (HSL) encoded by the *LIPE* gene (Sierra, 2004; Slominski et al., 2015).

Brain cholesterol, however, originates mainly from local synthesis in neuronal and glial cells as the blood-brain barrier prevents the entry of circulating lipoproteins carrying cholesterol (Ito and Yokoyama, 2003; Jeske and Dietschy, 1980). Brain cholesterol represents 25% of body cholesterol, mainly (99.5%) in the unesterified form, and is present in 70% of myelin and 30% of the plasma membrane of astrocytes and neurons (Björkhem and Meaney, 2004). The steady-state concentration of cholesterol in the brain remains relatively constant under physiological conditions due to a tightly regulated synthesis and turnover process, with a pool fraction constantly being replaced (Zhang and Liu, 2015). Production of cholesterol from acetyl-CoA occurs in the ER of astrocytes and neurons, where the presence of the enzyme HMG-CoA reductase is the rate-limiting step in cholesterol synthesis (Elustondo et

al., 2017; Martín et al., 2014; Zhang and Liu, 2015) (Figure 1). In addition, cholesterol import into neurons may occur with the uptake of apolipoprotein E-containing cholesterol from astrocytes, which is then converted to free cholesterol in endosomes/lysosomes. Excess cholesterol in cells can be regulated in several ways. One turnover in neurons involves the esterification/hydroxylation of cholesterol with a storage in lipid droplets as an esterified form. The major metabolic pathway of brain cholesterol, which occurs primarily in neurons, is the production of 24S-hydroxycholesterol by cholesterol 24-hydroxylase encoded by *CYP46A1* and its excretion into plasma by crossing the blood-brain barrier (BBB). Cholesterol can also be released from the cell as a complex with apolipoprotein-containing lipoprotein via the ATP-binding cassette (ABC) transporter at the membrane of astrocytes and neurons (Martín et al., 2014; Zhang and Liu, 2015).

The intracellular trafficking of free cholesterol in steroidogenic cells has not been fully elucidated. Transfer of free cholesterol to mitochondria may involve several pathways, including direct interactions between the mitochondria and intracellular organelles (ER, lipid droplets, lysosomes/endosomes), transport by cytosolic proteins, and interaction with a multi-protein complex at the OMM (Figure 1). Coordination processes for cholesterol mobilization to the OMM may involve the steroidogenic acute regulatory (StAR) protein in conjunction with the translocator protein (18 kDa) TSPO (formerly known as peripheral benzodiazepine receptor, PBR) together with TSPO-associated proteins (Papadopoulos et al., 2006; Papadopoulos and Miller, 2012). It has been proposed that these proteins form a complex and function as an outer mitochondrial signaling complex. This multi-protein complex includes OMM and IMM proteins and may be involved in the interaction with the IMM enzyme, P450scc (for the cleavage of cholesterol to pregnenolone) (Papadopoulos et al., 2015). In the brain, StAR is widely distributed in specific populations of neurons and glial cells, and StAR expression has been found in pyramidal neurons, granule cells, and the dentate gyrus of the

hippocampus. Neuronal and glial staining revealed a much wider distribution of StAR-positive cells in the striatum, cerebellum, pons and thalamus. StAR protein was also detected, to a lesser extent, in neurons in the preoptic area and in the arcuate nucleus of the hypothalamus (Sierra, 2004). In addition, co-expression of StAR and the enzyme P450scc has been demonstrated in neuronal subpopulations in mouse and human brains (King et al., 2002). Since StAR-mediated transport appears to be low under basal conditions, it has been proposed that plasma membrane cholesterol may also be transported by START (StAR-related lipid transfer) proteins, which contain a lipid-binding domain homologous to the C-terminus of StAR (Reitz et al., 2008). This homology led to the renaming of StAR to STARD1 for steroidogenic acute regulatory protein-related lipid transfer domain containing protein 1 (Elustondo et al., 2017). Several START domain proteins, such as STARD4, have been proposed to play a role in intracellular cholesterol transfer, but several lines of evidence, such as the absence of alterations in steroidogenesis in StarD4 knockout mice, support a role for STARD4 family proteins in cholesterol delivery to the OMM in most cell types, although possibly not in steroidogenic cells (Miller and Auchus, 2011).

Along with StAR, TSPO is another potential member of the cholesterol transport protein complex. TSPO is a ubiquitous mitochondrial protein localized in the OMM, abundant in steroidogenic tissues and well conserved across species (Papadopoulos and Miller, 2012). A consensus cholesterol recognition amino acid sequence (CRAC) has been identified in the C-terminal region of TSPO/PBR (Georges et al., 2021; Li et al., 2001), which is consistent with the increase in pregnenolone production in response to TSPO drug ligands in *in vitro* models and in rats (Lejri et al., 2019; Liere et al., 2017; Papadopoulos et al., 2015, 2006), as well as its decrease following TSPO mutations in rats (Owen et al., 2017). However, the role of TSPO in steroidogenesis has been challenged (El Chemali et al., 2022). As an illustration, it was recently shown that the absence of TSPO in complete TSPO/PBR knock-out mice did not

alter the steroidome content, including pregnenolone, in adrenals, testes, plasma and brain under basal conditions, although cholesterol transport was not impaired (Liere et al., 2023).

Following intracellular transport to the OMM, cholesterol is transferred from the OMM to the IMM (Poderoso et al., 2009) and converted to pregnenolone. The structural organization of cholesterol, in particular the C20-C22 region of its side chain, allows recognition and binding to the catalytic domain of the enzyme P450scc. P450scc is involved in three consecutive reactions to convert cholesterol to pregnenolone, including the hydroxylation of C22 and C20 and the subsequent cleavage of the C22-C20 bond. Expression of the P450scc enzyme has been demonstrated in brain astrocytes and neurons (Le Goascogne et al., 1987; Warner and Gustafsson, 1995) and in several rodent brain regions, including the cortex, amygdala, hippocampus and midbrain (Kimoto et al., 2001; King et al., 2002). Human P450scc is encoded by a single gene on chromosome 15, the CYP11A1 gene (Chung et al., 1986). Its expression has been reported in the human temporal lobe, frontal lobe, hippocampus and cerebellum (Lin and Papadopoulos, 2021), but is much lower than in the adrenal glands, which express high levels of CYP11A1 mRNA (Stoffel-Wagner, 2003). Recently, a novel mitochondrial CYP450, CYP1B1, appears to be involved in pregnenolone synthesis in the brain, as its inhibition significantly reduced pregnenolone production in human glial cells, but not in human adrenocortical cells (Lin et al., 2023).

2.2.2. Neurosteroidogenesis

The innovative premise that steroids could be synthesized *de novo* "by the nervous system for the nervous system" (and so-called neurosteroids) (Baulieu, 1997; Corpéchot et al., 1981) has been well established over the past 30 years. The initial discovery of the neurosteroid concept was based on the detection of steroids in the brain of rodents several days after the removal of peripheral sources of steroids (Baulieu et al., 2001; Robel and Baulieu, 1994). In addition, steroid synthesis has been demonstrated in the spinal cord and peripheral nervous

system (Giatti et al., 2015; Melcangi et al., 2011; Mensah-Nyagan et al., 2008; Patte-Mensah et al., 2006).

Neurosteroids are synthesized in the peripheral and central nervous systems, mainly in glial cells and neurons, from cholesterol or from precursor steroids imported from peripheral sources (Agis-Balboa et al., 2006; Baulieu et al., 2001; Compagnone and Mellon, 2000; Melcangi et al., 2008; Shibuya et al., 2003). Evidence for neurosteroidogenesis has been provided by experimental research in non-mammalian and mammalian brains, as well as in human brain and cerebrospinal fluid (CSF). This concept has been further validated by studies of the expression of steroidogenic enzymes for synthesis and metabolism, and of neurosteroid content in brain tissues and cells. The main pathways of steroid synthesis and metabolism of pregnenolone into the five main classes of steroids, progestogens, mineralocorticoids, glucocorticoids, androgens and estrogens, have been described in the brain (Raux et al., 2022). Pregnenolone can be processed by several metabolic pathways, depending on the demands and needs of the cells. Firstly, pregnenolone can be converted either to progesterone or to 17-hydroxy-pregnenolone and DHEA. Pregnenolone and DHEA can be metabolized to 7α-hydroxylated derivatives via the action of the enzyme Cyp7B, which is present in the mammalian brain (Akwa et al., 1992; Morfin and Stárka, 2001; Rose et al., 1997; Stapleton et al., 1995). Another alternative is the sulfation of the C3 hydroxyl group of pregnenolone to a 3β-sulfated derivative by sulfotransferase enzymes in rodent and human brain (Smith et al., 2014). However, the presence of sulfated pregnenolone, which has been reported in postmortem human brain tissue, is controversial in the rodent brain (Schumacher et al., 2008).

We previously found that the level of pregnenolone sulfate (measured by RIA in collaboration with the laboratory of Prof E-E Baulieu, Le Kremlin-Bicêtre, France) in the hippocampus of aged male rats was inversely correlated with their cognitive performance (Vallée et al., 1997). Subsequent studies using highly sensitive steroid extraction and mass

spectrometry methods in several laboratories suggested that the brain pregnenolone sulfate measured was more likely to be lipid-bound pregnenolone and demonstrated the absence or very low levels of pregnenolone sulfate in the rodent brain (Higashi et al., 2003a, 2003b; Liere et al., 2004; Liu et al., 2003; Mitamura et al., 2005; Schumacher et al., 2008). These findings are consistent with the following observations: 1) the sulfated form of pregnenolone is unlikely to cross the BBB, which would require the presence of transporter proteins, as sulfated steroids are no longer lipophilic (Foster and Mueller, 2018; Qaiser et al., 2017); and 2) although pregnenolone sulfate could be taken up across the BBB in rodents, it would be rapidly metabolized in favor of free pregnenolone (Qaiser et al., 2017).

In addition, cholesterol sulfate has been detected in rodent brains (Ivanisevic et al., 2014; Liu et al., 2003; Vitku et al., 2022), suggesting that pregnenolone sulfate may be produced from cholesterol sulfate via the P450scc (Cyp11a1) enzyme. The 3β-sulfation of cholesterol and pregnenolone requires the sulfotransferase enzyme isoform SULT2B1 (Fuda et al., 2002), but there is still no consensus on whether this enzyme is present in the brain (Vitku et al., 2022). In addition, cholesterol sulfate, but not pregnenolone sulfate and DHEA sulfate, was detected in the same rat brain samples (Liu et al., 2003). On the other hand, steroid sulfatase (STS) is present in the BBB as well as in some human brain regions such as the thalamus, hypothalamus, hippocampus and temporal lobe (Steckelbroeck et al., 2004). Further studies are needed to elucidate the role of sulfated steroids in the rodent and human brain, as imbalances in steroid sulfation/desulfation have been implicated in neurodegenerative diseases (Vitku et al., 2022).

Another metabolic pathway from pregnenolone to progesterone requires the enzyme 3β-hydroxysteroid dehydrogenase (3β-HSD), which is expressed in the rat and human brain (Brown et al., 2000; Guennoun, 2020; Guennoun et al., 1995; Schumacher et al., 2004; Yu et al., 2002). Two enzymes, 5α-reductase and 3α-hydroxysteroid dehydrogenase (3α-HSD) or

3α-hydroxysteroid oxido-reductase (3α-HSOR), which convert progesterone to its active metabolite, allopregnanolone (3α,5α-tetrahydroprogesterone), have been localized in major glutamatergic output neurons in the cortex, hippocampus and amygdala of mice and in the human brain. The conversion of pregnenolone to DHEA in the brain by 17-hydroxylase/17,20-lyase (P450c17) is challenging because no expression of P450c17 has been detected in rat brain glia (Cascio et al., 2000), but adult rat brain and neurons and astrocytes in culture have been shown to convert pregnenolone to DHEA (Akwa et al., 1993; Schumacher et al., 2008). In the human brain, postmortem studies reported that P450c17 was not expressed in the temporal lobe and limbic system of adult humans (Steckelbroeck et al., 2001), but was present in the developing human fetal nervous system, suggesting that DHEA may be synthesized locally (Schonemann et al., 2012). Finally, in the rat and human brain, DHEA can be converted to androstenedione and then to testosterone, which can be metabolized by aromatase to estradiol (Melcangi et al., 1998; Steckelbroeck et al., 2001; Stoffel-Wagner, 2003). Similarly, there is evidence that estradiol can be synthesized from pregnenolone in the rat hippocampus (Hojo et al., 2004).

In addition to the multitude of pathways, the complexity and specificity of steroid metabolism in a given species also depends on the specific expression of steroidogenic enzymes in tissues and/or cells. For example, the delta 5 (involving the metabolism of pregnenolone to 17OH-pregnenolone) and delta 4 (involving the metabolism of pregnenolone to progesterone and then to 17OH-progesterone) pathways are predominant in the gonads of rodents and humans, respectively (Connan-Perrot et al., 2021). It should be noted that the same enzymes may be involved in several pathways, suggesting that the study of their expression is not sufficient to identify the main pathways involved, but also requires the determination of steroid content.

2.2.3. Other sites of steroid production

Other tissues have also been classified as steroidogenic tissues because they express at least the enzyme P450scc, which initiates steroid synthesis by converting cholesterol to pregnenolone. Among them, adipocytes, intestine, gut, skin, retina and muscles have been reported as sites of steroidogenesis in mammals (see Graphical Abstract). Indeed, the presence of mitochondrial cholesterol transport and metabolism machinery, including STAR and/or P450scc, suggests that adipocytes (Byeon and Lee, 2016; Connan-Perrot et al., 2021; Li et al., 2014), gastrointestinal tissues (Bouguen et al., 2015; Mohibbi et al., 2017), skin (Hannen et al., 2011; Slominski et al., 2015, 2021), retina (Cascio et al., 2015; Jaliffa et al., 2005) and skeletal muscle (Vechin et al., 2023) have the ability to synthesize pregnenolone *de novo*. In addition, most of the enzymes for the conversion of pregnenolone to other steroids have been discovered in mouse and human adipose and intestinal tissues (Bélanger et al., 2002; Bouguen et al., 2015; Li et al., 2014; Sidler et al., 2011; Tchernof et al., 2015). Recently, the expression of enzymatic steps of steroidogenesis (StAR, P450scc, 3β-HSD, 5α-reductase, 3α-HSOR and aromatase) and the content of steroids, including pregnenolone, DHEA, progesterone and testosterone, and their metabolites were detected in the colon of adult male rats, which may play a role in the brain-gut axis function (Diviccaro et al., 2022, 2020). These findings provide additional insight into the distribution of steroids and the relationships that may exist between the different tissues of steroidogenesis, and warrant further studies of the steroid metabolome associated with specific physiological and/or pathological conditions.

2.3 Neuromodulation and neurosteroid molecular targets: specificity of pregnenolone

2.3.1. Genomic and non-genomic actions of steroids

The distinction between genomic and non-genomic functional activity of steroids lies in the differences in onset of action and localization of target receptors and intracellular

trafficking (see Graphical abstract). Classically, the delayed (usually hours or days) biological processes of steroids have been associated with nuclear steroid receptors and transcriptional regulation (Beato et al., 1996; Cole et al., 2019; Evans, 1988; Levin and Hammes, 2016; Truss et al., 1992). In this context, steroid signaling refers to steroid receptors located as monomers in the cytoplasm (as for androgen and glucocorticoid receptors) or in the nucleus (as for estrogens). The dimerized nuclear receptors then bind to specific steroid response elements (SREs) and can interact with various co-regulators or bind to other transcription factors (such as activating protein-1, AP-1 or specific protein-1, SP-1) to modulate (either repress or activate) the transcription of specific genes. In contrast to this transcription-dependent genomic effect, steroids have also been described to have faster (minutes, even seconds) and shorter non-genomic effects, probably mediated via specific membrane receptors. One of the earliest indications of a non-genomic mechanism of action of steroids was 80 years ago (with the first study on the anesthetic effects of steroids) (Selye, 1942) that steroids could elicit rapid responses that could not be explained by gene regulation. Since then, there has been increasing evidence that several steroid receptors actually reside in extranuclear cellular pools, including the plasma membrane (McEwen, 1991), and are involved in mediating rapid cell signaling that affects many aspects of cell biology (Levin and Hammes, 2016). It is important to note that the effects of steroids may be mediated by a multi-targeted mode of action via genomic and non-genomic pathways, which may influence each other and have complementary effects (Colciago et al., 2020; Szczurowska et al., 2022; Wilkenfeld et al., 2018).

Recently, the exploration of the mechanisms involved in the neuromodulatory effects of neurosteroids, especially their non-genomic actions, has opened a new frontier in neuroendocrinology. This research includes the effects of steroids on the dynamics of single membrane receptor molecules and steroid-membrane interactions in model lipid membranes

(Atkovska et al., 2018; Barabás et al., 2018; Crowley et al., 2022). Barabás and co-workers used single-molecule tracking technology, which allows real-time recording of the movements of individual molecules on receptors in the cell membrane. They reported that steroids can interfere with the surface trafficking of membrane receptors, which subsequently determines their docking and ligand binding properties and hence downstream signaling (Barabás et al., 2018). These concepts were illustrated using the effects of glucocorticoids and estradiol on the surface diffusion of glutamate and $GABA_A$ receptors, respectively.

In a complementary approach, Atkovska and colleagues reported that the interaction of steroids with phospholipid membranes can influence their binding to receptors (Atkovska et al., 2018; Crowley et al., 2022). Depending on the location of the binding site on the membrane receptors, the binding of steroids can be controlled by several factors, including their insertion properties (orientation and insertion depth) and the kinetics of the transition (flip-flop and exit) at the membrane (Atkovska et al., 2018). Steroids, being hydrophobic, can directly interfere with phospholipid bilayers and membranes. Interestingly, although steroids share a common structural tetracyclic core, they differ in their interaction with lipid membranes, mainly due to the type of functional groups at the C-3 and C-17 positions, representing the head and tail of the steroid, respectively.

The position and orientation of steroids in the membrane are therefore key factors in their functional activity. Indeed, the activity of steroids at specific membrane receptor targets depends, among other factors, on the conformation of the steroids, including their orientation and insertion depth, both of which are related to the ability of the steroid functional groups to form hydrogen bonds (h-bonds) with the lipid membrane. In addition, the orientation of the steroids determines their ability to approach and dock to membrane-embedded binding sites. Thus, a favorable or unfavorable conformation of the steroid results in its high or low activity. For example, the presence of a hydroxyl (OH) group on C-3 (head) and an acetyl group on C-

17 (tail) for pregnenolone facilitates and reduces the h-binding capacity, respectively. Therefore, the orientation of pregnenolone is favored in a vertical orientation with the tail inserted into the hydrophobic core and the head interacting with the head lipid groups of the phospholipid membrane (Atkovska et al., 2018). Furthermore, the authors reported that the kinetics of the steroid transition at the phospholipid membrane depends on the flip-flop and exit rates, which can be calculated as a function of the number of hydroxyl and carbonyl groups in the steroids. For example, the flip-flop rate is inversely proportional to the number of hydroxyl groups.

In addition to the direct interaction of steroids with membrane receptors, non-genomic pathways may involve indirect mechanisms associated with the binding of steroids to the phospholipid membrane, which, at sufficiently high concentrations, may lead to changes in membrane structure or fluidity that can subsequently affect the receptor (Crowley et al., 2022).

The described steroid-phospholipid membrane dynamics were based on a model lipid membrane and should be extended to a more complex membrane cell in order to establish a reliable structure-activity relationship in steroid-membrane interactions, which could be useful in determining the physiological mechanism of action and potency of steroids (Crowley et al., 2022). Indeed, steroid-induced changes in membrane properties may be related to steroid concentration and lipid composition, as exemplified by the concentration-dependent decrease in bilayer thickness upon the addition of cortisone, which can be reduced in the presence of cholesterol (Alsop et al., 2016; Khondker et al., 2019). Notably, in the above biophysical studies, steroid concentrations were used at levels corresponding to the increased local and temporal steroid concentrations achieved with pharmacological treatment.

2.3.2. The concept of neuroactive steroids and neurosteroids: an original class of messengers in the brain

The term 'neuroactive steroids', coined by Paul and Purdy (Paul and Purdy, 1992), refers to steroid-based compounds of both natural and synthetic origin that can act rapidly on brain excitability at the membrane level without interacting with classical intracellular steroid receptors, but rather bind stereoselectively and with high affinity to membrane receptors (McEwen, 1991; Rupprecht, 2003; Tuem and Atey, 2017). It has been shown that neurosteroids and neuroactive steroids can act as neuromodulators to regulate brain-related functions (Compagnone and Mellon, 2000; Longone et al., 2011). Therefore, the terms neurosteroids and neuroactive steroids can be used interchangeably, although the term neuroactive steroids has also been used as an umbrella term to include both exogenous and endogenous steroids, including hormonal steroids and neurosteroids (Marwein et al., 2020).

The term neurosteroids may therefore be preferred to refer to both their synthesis in the central nervous system and their neuromodulatory action via membrane receptors. Neurosteroids have thus been described as the 4th generation of messengers in the brain because of their rapid modulation of communication between neurons via neurotransmitter receptors (Kawato et al., 2003; Shibuya et al., 2003). Previous generations of neurotransmitters include first-generation neurotransmitters such as glutamate, GABA and acetylcholine, second-generation catecholamines (dopamine and serotonin) and some third-generation neuropeptides such as enkephalin and substance P. Unlike the first three groups of messengers, which are stored in vesicles and released from presynapses, neurosteroids are produced in mitochondria and microsomes and are released into cells by passive diffusion due to their lipophilic properties. Neurosteroids can therefore have autocrine and paracrine effects, acting in the same cell or in neighboring cells.

2.3.3. Classical molecular targets and major neuromodulating effects of neurosteroids

The non-genomic actions of neurosteroids have been best described through the modulation of two ionotropic membrane receptors, the GABA (gamma-aminobutyric acid) A receptors and the NMDA (N-methyl-D-aspartic acid) class of glutamate receptors (see Graphical abstract) (Belelli et al., 2002; Belelli and Lambert, 2005; Irwin et al., 1994; Lambert et al., 2009; Majewska, 1992; Rupprecht, 2003; Sedlácek et al., 2008), which are two of the major inhibitory and excitatory neurotransmitter receptor families involved in synaptic transmission in the brain (Sears and Hewett, 2021). Thus, two families of neurosteroids can emerge that act on the balance between inhibitory and excitatory brain inputs, based mainly on the chemical structure and conformation of the steroid (Park-Chung et al., 1999; Weaver et al., 2000) and the subunit composition of the receptor (Belelli et al., 2022; Cameron et al., 2012). Among the inhibitory neuromodulators, allopregnanolone remains the most studied, as it is a potent positive allosteric modulator of the $GABA_A$ receptor that shows high potency at known binding sites (Hosie et al., 2006), resulting in rapid and physiologically relevant neuromodulatory control of brain excitability.

A functional dichotomy has therefore been reported between the effects of excitatory, such as the sulfated derivatives of pregnenolone and DHEA (PREG-S and DHEA-S), and inhibitory (such as allopregnanolone) neurosteroids. Briefly, the former have mainly pro-memory and anxiogenic properties, whereas the latter have more amnesic, anxiolytic and antidepressant effects (Alomary et al., 2003; Barbaccia et al., 2001; Brot et al., 1997; Khisti et al., 2000; Mathis et al., 1994; Tomaselli and Vallée, 2019; Vallée et al., 2003). In addition, synthetic analogs of PREG-S that differ in their modulatory effects on NMDA and $GABA_A$ receptors have different effects on memory in adult mice (Alomary et al., 2003; Vallée et al., 2001a).

Other receptor targets have been described for neurosteroids, including the AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) subtype of ionotropic glutamate membrane receptors and the intracellular $sigma_1$ receptor (Maurice et al., 2001). $Sigma_1$ receptors are mainly localized to the membrane of the endoplasmic reticulum (ER) and at the level of the cholesterol-rich region of the ER mitochondria-associated membrane (MAM) (Hayashi, 2015; Mtchedlishvili and Kapur, 2003; Schverer et al., 2018).

2.3.4. Molecular targets and major neuromodulating effects of pregnenolone

Unlike other neurosteroids, pregnenolone has little or no affinity for $GABA_A$, NMDA or $sigma_1$ receptors (Gibbs et al., 2006; Maurice et al., 2006; Park-Chung et al., 1999; Weaver et al., 2000), which has long justified its designation as an inactive precursor steroid (or proneurosteroid).

Hence, the functional activity of pregnenolone has been poorly studied, but some studies have reported that pregnenolone administration can modulate animal behavior (Eser et al., 2008; Vallée, 2016; Zheng, 2009). For example, pregnenolone has antidepressant-like effects and dose-dependent anxiogenic or anxiolytic effects in rodents (Eser et al., 2008; Melchior and Ritzmann, 1994; Reddy and Kulkarni, 1998). In addition, pregnenolone can induce cognitive improvements in avoidance, food-motivated and working memory paradigms in mice and in a mouse model of schizophrenia (Flood et al., 1992; Isaacson et al., 1994; Melchior and Ritzmann, 1994; Wong et al., 2012). Pregnenolone may also have neuroprotective effects. Pregnenolone, like other neurosteroids, can be synthesized in Schwann cells (Leonelli et al., 2006; Melcangi et al., 2008; Patte-Mensah and Mensah-Nyagan, 2008; Schumacher et al., 2004) and plays a regulatory role during myelination (Zhu and Glaser, 2008). Furthermore, in spinal cord injury, pregnenolone can reduce histopathological consequences, protect nerve tissue from secondary damage and improve recovery of motor function (Guth et al., 1994).

In the 2000s, a specific intracellular target for pregnenolone was discovered at the cytoplasmic microtubule site in brain extracts and *in vitro* (Mizota and Ueda, 2008; Murakami et al., 2000). Pregnenolone, together with DHEA, can bind and activate the microtubule-associated protein MAP2 and the microtubule-binding protein CLIP-170 (Fontaine-Lenoir et al., 2006; Weng et al., 2013), families of proteins that are mainly expressed in neuronal cell bodies and dendrites, where they modulate the assembly and stabilization of microtubules and their growth during cell migration (Conde and Cáceres, 2009). In addition, *in vitro* studies have shown that pregnenolone can bind to the protein shugoshin 1 (sSgo1), which protects centriole cohesion (at the microtubule organization center of the cell) during mitosis (Hamasaki et al., 2014). Functional implications have been proposed for a synthetic derivative of pregnenolone, 3β-methoxypregnenolone (MAP4343), which acts on the MAP2 protein, with a protective role in spinal cord injury (Duchossoy et al., 2011) and against depression in animal models (Bianchi and Baulieu, 2012) and in humans (Barbiero et al., 2022; Daftary et al., 2017) (ClinicalTrials.gov identifier: NCT03870776).

In the 2010s, we discovered a novel mechanism of action of pregnenolone involving cannabinoid type 1 receptors (CB1Rs) (Vallée et al., 2014) (see Graphical abstract), which are part of the metabotropic class A G-protein coupled receptors (GPCRs) (Leo and Abood, 2021) and are the most highly expressed GPCRs in the brain (Howlett et al., 1990; Mechoulam and Parker, 2013). Computational experiments in the laboratory of Dr Patrizia Reggio (UNC Greensboro, North Carolina, USA), using the Forced-Biased Metropolis Monte Carlo (MMC) simulated annealing program for ligand-protein binding (Clark et al., 2006) in a CB1R model (Marcu et al., 2013), have shown that pregnenolone binds to a specific binding pocket formed by the transmembrane domains TMH1 and TMH7 associated with the C-terminal intracellular helix 8 (Hx8) (Vallée et al., 2014). Each polar group of pregnenolone interacts with residue E.1.49 (for the ketone group at C17) or R7.65 (for the hydroxyl group at

C3) on the TMH1,7 and Hx8 regions, respectively. The localization of this binding site was confirmed by an *in vitro* experiment measuring mitochondrial respiration in cells transfected with a mutant human CB1R (hCB1Rp.E1.49G) in which the glutamate (E) residue at position 1.49 has been replaced by glycine (G). In these cells, the inhibitory effect of pregnenolone on the THC-induced decrease in cellular respiration was absent, in contrast to what was observed in cells transfected with a wild-type hCB1R (Vallée et al., 2014).

By evaluating the molecular processes, we have interestingly shown that pregnenolone is an endogenous biased negative allosteric modulator of CB1R, opening a great functional and therapeutic opportunity (Vallée et al., 2014). Thus, an innovative endogenous neuromodulatory mechanism has been revealed with the emergence of a regulatory loop between pregnenolone and the endocannabinoid system involving CB1R regulation (Raux et al., 2022; Raux and Vallée, 2022). This regulatory process can occur in numerous brain regions that contain both P450scc (Cyp11a1) and CB1Rs. In the CNS, CB1Rs are expressed at high levels in regions responsible for mental and physiological processes, such as the basal ganglia (movement), cerebellum (motor coordination), hippocampus (memory), cerebral cortex (cognition), hypothalamus (appetite), and amygdala (emotions), and to a lesser extent in the brainstem (respiratory and cardiac control) (Rezende et al., 2023; Tao et al., 2020). CB1Rs are found primarily, though not exclusively, in presynaptic terminals where they control retrograde inhibition of neurotransmission and maintain the excitatory/inhibitory balance in the brain. Thus, CB1R expression is a fine-tuning system of homeostasis, and CB1 activation functions as an adaptive response to maintain homeostasis, but overstimulation of CB1Rs shifts them from their physiological roles to pathological states (Lutz, 2020). Therefore, the regulatory loop involving pregnenolone and CB1Rs is likely to arise in the adjustment of this imbalance. This is illustrated by the ability of pregnenolone to functionally reverse CB1-mediated outcomes associated with the toxic and psychotic effects of cannabis in

mouse models (Busquets-Garcia et al., 2017; Vallée et al., 2014), opening new therapeutic perspectives for pregnenolone (Vallée, 2016). However, to overcome the properties of pregnenolone, including short half-life, low oral bioavailability, and metabolism in downstream steroids, non-metabolized synthetic analogs of pregnenolone have been developed (www.aelisfarma.com). One of these, AEF0117, which targets the CB1R as a signaling specific inhibitor, was able to significantly reduce cannabis use and cannabis-related disorders in animal models and humans (Haney et al., 2023) (ClinicalTrials.gov identifiers: NCT03325595, NCT03443895 and NCT03717272).

3. New insights from metabolomics approach

3.1. From pharmacological action to physiological function

Treatment with endogenous molecules that are part of a metabolic pathway raises questions about the active metabolite responsible for the observed end-result. Therefore, to confirm the relationship between the pharmacological effects of exogenous neurosteroids and the physiological function of the specific neurosteroid, it is worthwhile to analyze the endogenous levels of the administered neurosteroid and its metabolites. In addition, the analysis of the profile of steroid metabolites (also known as the steroid metabolome) is even more promising when these metabolites may have different or even opposite effects, as is the case for the inhibitory and excitatory neuromodulation effects of some neurosteroids.

3.2. Neurosteroigenesis in biochemical research

The knowledge of neurosteroid synthesis pathways and their molecular targets has paved the way for the discovery of their functional role as endogenous modulators in the brain. It is therefore interesting to assess their endogenous levels in relation to a defined function in order to better understand their physiological significance. In other words, knowledge of the endogenous steroid profile is a prerequisite for detecting alterations in steroidogenesis in healthy and diseased neuronal structures (Melcangi et al., 2008). This could help biomedical

research to elucidate the mechanisms involved in alterations in neurosteroidogenesis and to discover candidate biomarkers for the diagnosis of brain disorders, leading to the development of potential therapeutic strategies. Consequently, the research strategy of our laboratory and others has been based on the validation of methods coupling mass spectrometry and gas chromatography. It has been selected as the method of choice for determining low concentrations of multiple steroids in the same biological sample, in addition to more specific methods for extracting steroids from biological tissues (Alomary et al., 2001; George et al., 2010; Matias et al., 2023; Vallée, 2014; Vallée et al., 2000). This technological development has allowed us to address some of the functional roles of neurosteroids, particularly for pregnenolone (Vallée et al., 2014). The basis for this strategy was the development of steroid research, which focused on the study of steroid metabolites rather than a single steroid.

3.3. From a single steroid to a metabolic profile: benefiting from technological advances

The concept that individuals might have a "metabolic profile" in their biological fluids was first introduced in the 1940s and then demonstrated in the 1970s by measuring compounds present in human urine and tissues (Novotny et al., 2008). Subsequently, the importance of metabolomics in determining changes in the brain profile of steroid metabolites in health and disease was emphasized (Griffiths and Wang, 2009; Melcangi et al., 2008). Metabolomics aims to identify and quantify a wide range of molecules (<1000 Da) in a sample. The classical steroid assay methods using immunoassays (IA), such as radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA) techniques, which are based on antibody-protein recognition, have some limitations for the quantification of steroid metabolites (Matias et al., 2023). First, ligand-binding assays are not available for all steroids. Second, when available, the antigen-antibody reaction presents a potential crossover between similar molecules, which in many cases reduces specificity and interassay reproducibility and leads to

potential false-positive results (Kaleta et al., 2021; Krassowski et al., 2020; Saitman et al., 2014). The existence of steroid isomers with the same chemical structure but a different spatial conformation, which may act differently and be involved in different functions, makes them difficult to identify using AI methods. For example, we and others have reported that PREG-S and its 3α-isomer affect adult rodent memory differently (Akwa et al., 2001; M. Vallée et al., 2001b; Vallée et al., 2003). In addition, endogenous allopregnanolone isomers (at the C-3 and/or C-5 positions) may differ in their effects on the $GABA_A$ receptor and related functions (Tateiwa et al., 2023; Wang, 2011). Finally, because IAs primarily target one biomolecule, there is a lack of information on related metabolites that may have different molecular and/or cellular targets than the molecule of interest. Consequently, the IA methodology does not support the evaluation of steroid pathways and networks in the same sample (Figure 2).

To overcome these challenges, mass spectrometry (MS)-based technologies have been developed and have challenged the field of analytical chemistry, to become an essential tool in metabolomics (Alseekh et al., 2021; Audano et al., 2021). The main advantages of MS techniques include the assessment of multiple molecules in a single biological sample during the same analytical run, allowing the assessment of a whole panel of compounds and pathway mapping (Figure 2). In addition, chromatographic separation, using gas chromatography and, increasingly, liquid chromatography coupled with MS, provides separation for the detection and measurement of similar molecules, such as isomers (Yu and Yao, 2017). Another advantage of this analytical technique is the use of internal standards, which provide high validity in terms of accuracy, precision and reproducibility (Duncan, 2017). Recently, advances in MS method development, such as tandem MS, have provided higher specificity along with improved sensitivity and specificity (Seger and Salzmann, 2020).

Advanced quantification methods have now been developed in several laboratories using state-of-the-art mass spectrometry coupled to chromatography methods, which allow the measurement of low concentrations of numerous steroids and metabolites (steroidome) in individual biological samples extracted from small rodent brain regions (Caruso et al., 2008; Concas et al., 2000; George et al., 2010; Griffiths and Wang, 2009; Higashi et al., 2005; Liere et al., 2023, 2017, 2000; Liu et al., 2003; Maldonado-Devincci et al., 2014a, 2014b; Marx et al., 2006b, 2006a; Marx et al., 2003; O'Dell et al., 2004; Park et al., 2017; Porcu et al., 2003; Uzunov et al., 1996; Vallée et al., 2014, 2000) and in human plasma, serum or cerebrospinal fluid (Caruso et al., 2015; George et al., 1994; Marx et al., 2006; Melcangi et al., 2013; Rasmusson et al., 2006; Ritsner et al., 2007, 2010; Romeo et al., 1996; Shibayama et al., 2009; Uzunova et al., 1998; Weill-Engerer et al., 2002).

3.4. Application of metabolomics in prognosis and medical diagnosis

Over the past decade, numerous reviews have been published on metabolomics for clinical purposes, and metabolomics has been described as the "stethoscope for the 21st century" (Ashrafian et al., 2021). Metabolic profiling is emerging as an advanced diagnostic technique that analyses the presence/absence of endogenous or exogenous molecules, as well as variations in metabolite levels due to phenotypic changes and/or environmental factors that may indicate the onset or presence of disease. In addition, this approach would allow the identification of metabolic fingerprints rather than a single metabolic biomarker, leading to the discovery of biomarkers predictive of therapeutic response. Thus, comparative profiling analyses between health and disease states may reveal biomarkers as indicators of biological changes. Furthermore, this metabolomics approach has recently been extended to the assessment of target and non-target metabolites, corresponding to a priori known and unknown endogenous molecules, respectively, which may allow for better disease diagnosis (Matias et al., 2023; Sharp et al., 2018).

4. Conclusion and perspectives

Advances in neuroendocrinology have been made possible by broadening our view of the regulatory loops between endogenous mediators, considering the brain first as a gateway and then as a key mediator. Indeed, a better understanding of steroid research has allowed us to elucidate additional sites of steroid production, including the brain, and new molecular targets involved in maintaining homeostasis in health and disease. This has recently been illustrated by neuromodulation through the interaction of pregnenolone with the CB1 receptor, which has led to innovative therapeutic beliefs in cannabis addiction and cannabis use disorders.

In addition, technological advances in mass spectrometry approaches are enabling the emergence of translational metabolomics, which offers a promising bridge between research laboratory data and clinical therapeutic applications.

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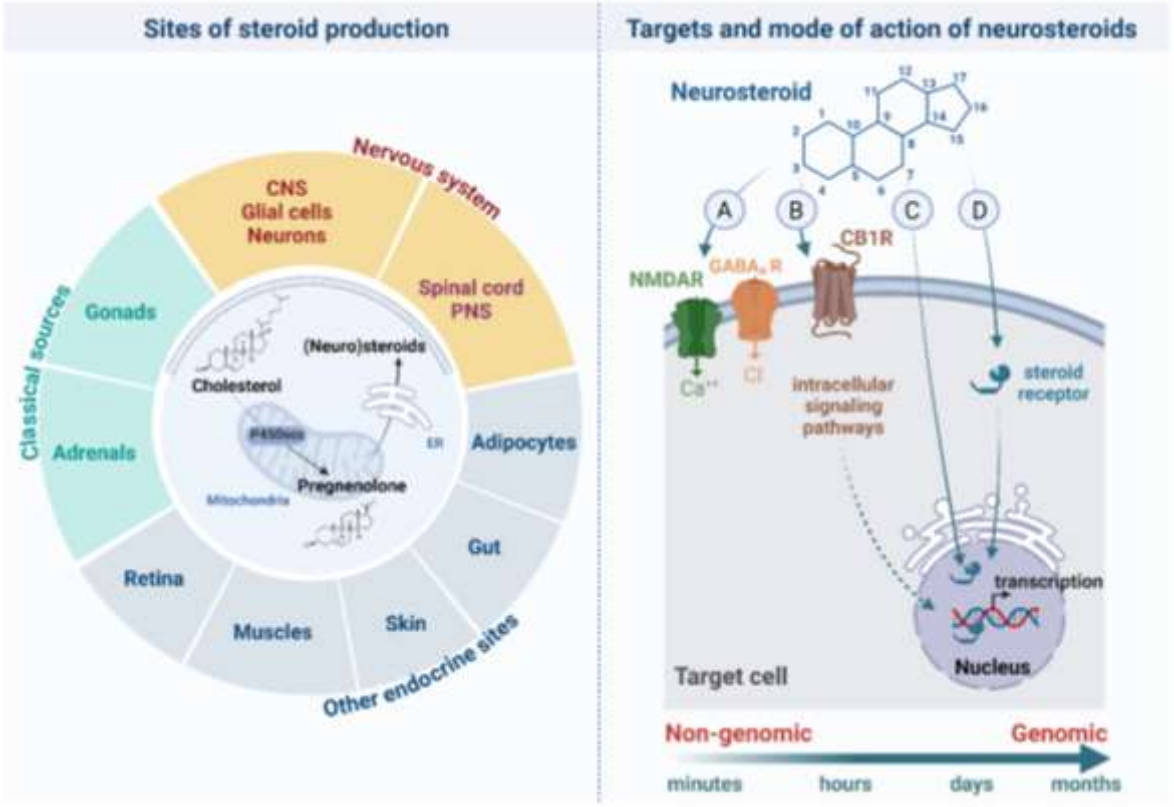
Table 1. Brief overview of major advances in neuroendocrine research

Concept / Illustration	Period	Main authors (pioneers)
Brain excretion of waste residue (*pituita*) distributed through the body by nerves	2nd century 16th century	Claudius Galenus Andreas Vesalius
Three-dimensional view of the cerebral ventricles	16th century	Leonardo da Vinci
Brain medial sagittal background of "Adam's creation" painting (Sistine chapel, Vatican, Italy)	16th century	Michelangelo Buonarroti
Basis of homeostasis	19th century	Claude Bernard Walter Bradford Cannon
Release of neuropeptides from the pituitary	early 20th century	Mary Pickford Berta Scharrer
Basis of hypothalamic-pituitary gonadal axis	mid-1950's	Geoffrey Harris
Steroid hormone feed-back onto the pituitary	1970's	Dora Jacobsohn Dorothy Price Martha Vogt
Hypothalamic peptide-releasing factors	1970's	Andrzej Wiktor Shally Roger Guillemin
Basis of hypothalamic-pituitary adrenal axis	1970's-1980's	Hans Selye Claude Fortier Wylie W. Vale
Assays of small molecules in fluids	1980's	Rosalyn Yalow
Neurosteroids	1980's-2000's	Etienne- Emile Baulieu Paul Robel Colette Corpéchot
Neuroactive steroids	1990's	Steven M. Paul Robert H. Purdy
Basis of GPCR molecular endocrinology	2010's	Brian Kobilka Robert Lefkowitz

GPCR, G-protein coupled receptors

Graphical abstract. Major advances in steroid research



Left. Steroidogenic tissues are characterized by the presence of the P450scc enzyme in the mitochondria, which mediates steroid synthesis by converting cholesterol to pregnenolone, the precursor of downstream steroids processed in the endoplasmic reticulum (ER). Classical sources of steroids include the adrenal glands and the gonads. Steroids are synthesized *de novo* in the central nervous system (CNS) in glial cells and neurons, as well as in the spinal cord and peripheral nervous system (PNS). In addition, other endocrine sites have been described, including adipocytes, gut, skin, muscles, and retina.

Right. The rapid non-genomic and delayed genomic effects of neurosteroids occur through membrane receptors and nuclear receptors, respectively. Membrane receptors classically include (A) the ionotropic NMDA receptors (as for PREG-S and DHEA-S) and $GABA_A$ receptors (as for allopregnanolone) and more recently (B) the G-protein coupled CB1 receptor (as for pregnenolone). Subsequent changes in intracellular signaling pathways can alter gene transcription. Nuclear receptors bind lipophilic steroids in the nucleus (C) (as for estrogens) or in the cytosol of the cell (D) (as for androgens and glucocorticoids) and translocate to the nucleus where they bind specific hormone response elements resulting in activation or repression of gene transcription.

CB1R, cannabinoid type 1 receptors; $GABA_AR$, gamma-aminobutyric acid A receptors; NMDAR, N-methyl-D-aspartic acid class of glutamate receptors

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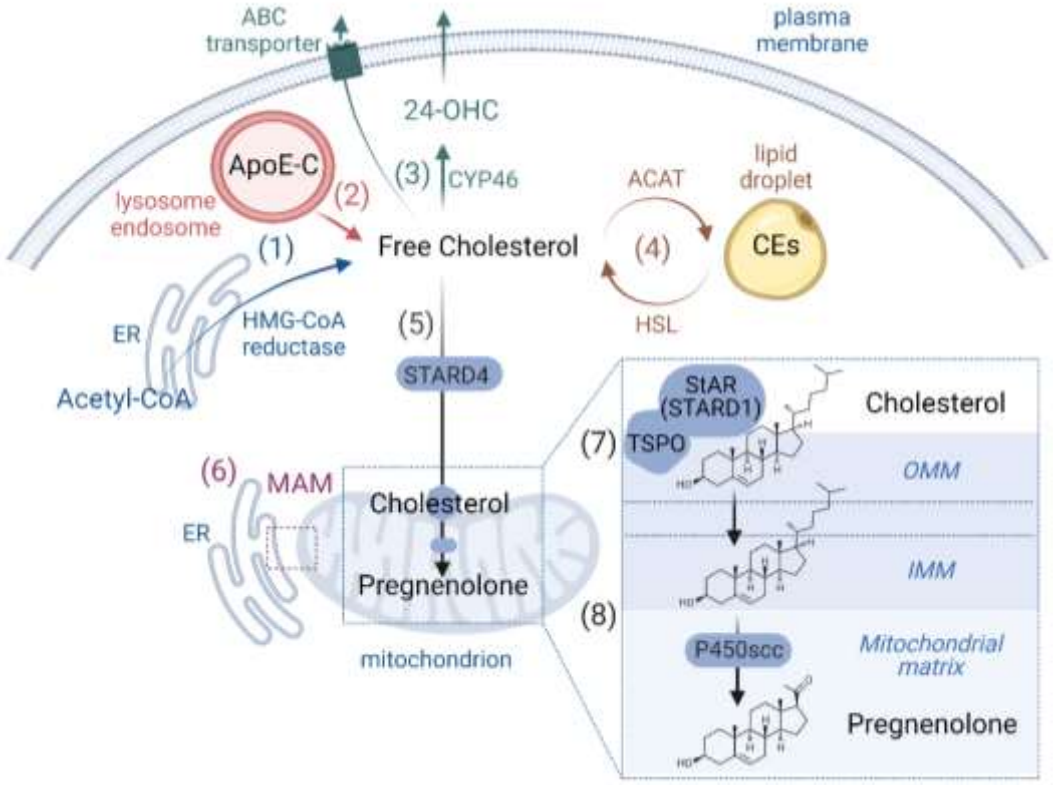
Figure 1. Cholesterol homeostasis and transport to mitochondria in steroidogenic brain cells.



Schematic representation of potential pathways of intracellular cholesterol homeostasis and trafficking, including (1) *de novo* synthesis of free cholesterol from acetyl-CoA in the endoplasmic reticulum (ER) via the enzyme HMG-CoA reductase; (2) uptake from apolipoprotein E-containing cholesterol (ApoE-C); (3) release via the ATP-binding cassette (ABC) membrane transporter or metabolism to 24-hydroxycholesterol (24-OHC) via the enzyme cholesterol 24-hydroxylase (CYP46); (4) esterification of excess cholesterol via the acyl-CoA Cholesterol Acyltransferase (ACAT) for storage as cholesteryl esters (CEs) in lipid droplets and hydroxylation by hormone-sensitive lipase (HSL); (5) cytoplasmic transport to mitochondria via the STARD4 protein; (6) import into the mitochondrion via mitochondria-associated membranes (MAM) at the ER; (7) interaction with the OMM via a multi-protein complex including StAR (also called STARD1) and TSPO proteins, (8) transfer from the OMM to the IMM and conversion to pregnenolone via the cholesterol side chain cholesterol cytochrome (P450scc) (*CYP11A1*) in the mitochondrial matrix..

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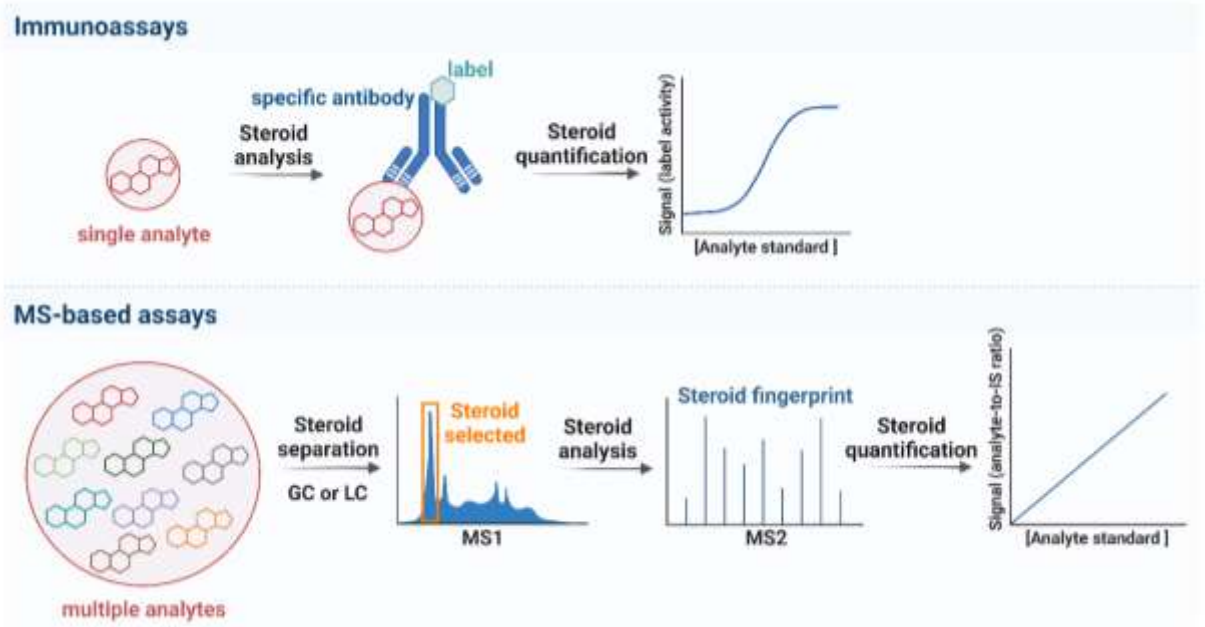
Figure 2. From single steroid analysis to metabolomics



Schematic of steroid assays using classical immunoassays (top) and mass spectrometry (MS)-based assays (bottom). Immunoassays involve the analysis of a single analyte via interaction with a specific labeled antibody, while MS methods coupled to gas or liquid chromatography (GC or LC) involve the analysis of multiple analytes in a sample. Steroid separation by GC or LC is followed by steroid analysis by MS, resulting in a specific steroid fingerprint for each selected steroid. Steroid quantification is based on an analyte calibration curve obtained for increasing concentrations of analyte standards from the label activity for immunoassays and from the analyte-to-internal standard (IS) ratio of peak intensities for MS methods.

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