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Review

Analytical challenges for measuring steroid responses to stress, neurodegeneration and injury in the central nervous system

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ABSTRACT

Levels of steroids in the adult central nervous system (CNS) show marked changes in response to stress, degenerative disorders and injury. However, their analysis in complex matrices such as fatty brain and spinal cord tissues, and even in plasma, requires accurate and precise analytical methods. Radioimmunoassays (RIA) and enzyme-linked immunosorbent assays, even with prepurification steps, do not provide sufficient specificity, and they are at the origin of many inconsistent results in the literature. The analysis of steroids by mass spectrometric methods has become the gold standard for accurate and sensitive steroid analysis. However, these technologies involve multiple purification steps prone to errors, and they only provide accurate reference values when combined with careful sample workup. In addition, the interpretation of changes in CNS steroid levels is not an easy task because of their multiple sources: the endocrine glands and the local synthesis by neural cells. In the CNS, decreased steroid levels may reflect alterations of their biosynthesis, as observed in the case of chronic stress, post-traumatic stress disorders or depressive episodes. In such cases, return to normalization by administering exogenous hormones or by stimulating their endogenous production may have beneficial effects. On the other hand, increases in CNS steroids in response to acute stress, degenerative processes or injury may be part of endogenous protective or rescue programs, contributing to the resistance of neural cells to stress and insults. The aim of this review is to encourage a more critical reading of the literature reporting steroid measures, and to draw attention to the absolute need for well-validated methods. We discuss reported findings concerning changing steroid levels in the nervous system by insisting on methodological issues. An important message is that even recent mass spectrometric methods have their limits, and they only become reliable tools if combined with careful sample preparation.

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Abbreviations: ACTH, adrenocorticotropic hormone; AD, Alzheimer's disease; BBB, blood–brain–barrier; CNS, central nervous system; CRH, corticotropin-releasing hormone; CSF, cerebrospinal fluid; DHEA, dehydroepiandrosterone; DHEAS, dehydroepiandrosterone sulfate; EAE, experimental autoimmune encephalomyelitis; 5α-DHP, 5α-dihydroprogesterone; ELISA, enzyme-linked immunosorbent assays; ER, estrogen receptor; $GABA_A$ receptors, gamma-aminobutyric acid type A receptor; GC, gas chromatography; HPA, hypothalamo-pituitary–adrenal; HPLC, high-performance liquid chromatography; 3α-HSD, 3α-hydroxysteroid dehydrogenase; 3β-HSD, 3β-hydroxysteroid dehydrogenase; LC, liquid chromatography; MCAO, middle cerebral artery occlusion; MDR, multidrug-resistance transporters; MS, mass spectrometry or multiple sclerosis; MS/MS, tandem mass spectrometry; NMDA, N-methyl-D-aspartate; OPC, oligodendrocyte progenitor cells; PR, progesterone receptor; PREGS, pregnenolone sulfate; PTSD, posttraumatic stress disorder; RIA, radioimmunoassay; SPE, solid phase extraction; SSRI, selective serotonin reuptake inhibitors; TBI, traumatic brain injury; THDOC, tetrahydrodeoxycorticosterone; TSPO, Tanslocase 18 kDa.

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1. Introduction

It has taken time to free steroids from their confined roles as reproductive or stress hormones, and it is now well established that they exert multiple functions throughout the CNS. Steroids that are derived from cleavage of the cholesterol side chain by cytochrome P450scc in mitochondria regulate a wide range of neuronal and glial functions by using multiple signaling mechanisms. Over the past years, particular attention has been paid to the neuroprotective and rescue actions of steroids after injury or during disorders of the CNS [1–8].

Steroid hormones play a major role during development of the CNS. Estrogens, progestogens and androgens indeed promote the maturation of neuronal circuits and the elaboration of myelin [9–16]. They are also involved in the protection of the immature grey and white matters during early stages of development, when the CNS is particularly sensitive to oxygen and glucose deprivation, injury and other types of stressors [17–21]. In the adult CNS, endogenous steroid signaling has been proposed to be continuously part of protective, rescue and regenerative processes [1,4]. This concept is consistent with the observation that developmental processes are recapitulated, although not always entirely, when adult neural cells respond to injury or degeneration and during repair processes [22–24].

Adaptive responses of neural cells to environmental challenges, neurodegenerative processes and injury are indeed reflected by changes in steroid levels within the CNS. However, their interpretation is complicated by the multiple sources of steroids: the steroidogenic endocrine glands and the local synthesis of neurosteroids by neurons and glial cells. Another difficulty is the accurate measure of low levels of the lipophilic steroid molecules, which only differ by the presence or absence of functional groups and double bonds in their ring structures, together with the unequivocal distinction between multiple stereoisomers. This is particularly challenging for complex matrices such as the very fatty brain and spinal cord tissues. The appraisal of changes in plasma and CNS steroid levels reported in the scientific literature thus requires an understanding of the principles and limitations of the available analytical methods. The lack of specificity of immunological methods for steroid analysis has always been a major problem and has contributed to the accumulation of inconsistent results in the literature. Currently, gas or liquid chromatography (GC or LC) coupled to mass spectrometry (MS) or tandem mass spectrometry (MS/MS) are considered as the gold standards for accurate and sensitive steroid analysis. Although it is generally believed that steroid measures obtained by mass spectrometric methods are always accurate and precise, an appeal to caution has to be made. It is important to be aware that these sophisticated analytical technologies involve many purification steps, which can become major sources of errors. Only when combined with carefully validated sample workup, mass spectrometric methods become robust analytical tools, providing accurate reference values for steroids in different tissues and under various pathophysiological conditions [25]. We thus urge a more critical awareness of analytical procedures for steroids used in experimental research and clinical studies.

2. Analytical methods for the determination of steroid levels: radioimmunoassays

Before discussing changes in CNS steroid levels in response to stress, injury and degenerative processes, challenges faced by accurate steroid analysis will be examined. Prior to the advent of radioimmunoassays (RIA), the analysis of hormones relied on colorimetric- and bioassays. The first use of a RIA in 1959 was for the measure of plasma insulin [26]. Only 10 years later, the first RIA of a steroid hormone, estradiol, was reported [27]. It was soon followed by the radioimmunological analysis of progesterone and pregnenolone [28,29]. One difficulty to the analysis of steroid hormones by RIA is their low molecular weight, which means that they are not immunogenic. The solution to this problem has been brought by linking covalently steroids to a protein. This combination then becomes immunogenic and behaves as haptens, allowing antibodies with a certain degree of specificity to be produced by active immunization [30].

Steroid RIA is based on the competition for a limited amount of antibody between the hormone to be quantified and a fixed amount of the corresponding hormone labeled with a radioisotope. After separation of the antibody-bound hormone from the free hormone, the amount of steroid in the biological sample is determined against a standard curve [30]. In the 1970s, most steroid RIA utilized an iodinated ligand, and a second antibody was often employed to separate antibody-bound ligand from unbound. Although the limited specificity of RIA for steroid analysis has always been a problem, it should be acknowledged that a great deal of our information concerning the physiologic and pathophysiologic roles of steroid hormones comes from studies that utilized validated RIA with preceding purification and separation steps (indirect RIA). In the past, many journals were strict about the careful validation of RIA procedures. Unfortunately, the requirement for properly validated RIA has become less severe. Even reference journals now publish steroid measures performed by direct commercial RIA or enzyme immunoassay kits "according to the instructions provided by the manufacturer". Although commercial kits are easy to use and less time consuming than well validated assays, they cannot guarantee accurate and robust analysis of steroids in biological samples, and in particular in complex matrices such as nervous tissues. It is indeed important to pay attention to the rigorous validation of RIA procedures and to the sample workup procedures [31–33].

As steroid hormones are converted in their target tissues to large numbers of metabolites, it is absolutely necessary to use separation steps by chromatography prior to RIA [33]. It is worth noting that even for the first reported steroid RIA, a pre-purification and

pre-separation step by Celite or Sephadex column chromatography was carried out to remove potentially interfering metabolites and other compounds [28,29,34]. Celite chromatography prior to RIA has indeed been shown to reduce levels of steroids measured in biological samples [35]. However, although such separation procedures improve the accuracy of RIA assays, they are not sufficient and limited specificity due to cross-reactions will always remain a serious issue when using immunological methods [36,37]. Even conventional RIA generally yield much higher levels of steroids in biological matrixes than mass spectrometric methods because of cross-reactions, especially for the low range of concentrations (picomolar) [35,38]. Moreover, for all assays based on the use of antibodies, analyte measurement only represents a surrogate approach, measuring radioactivity rather than the steroid itself.

Thus, even indirect RIA with the removal of interfering metabolites by Celite or Sephadex columns, together with the use of reasonably specific antibodies, do not offer sufficient selectivity for the accurate measure of steroid hormones, and especially for their very large number of stereoisomers (see Fig. 1 for the large, but not exhaustive number of progesterone metabolites and their stereoisomers identified in biological samples by mass spectrometric methods). The accurate analysis of steroids and their isomers indeed requires mass spectrometric methods preceded by GC or LC. For example, levels of estradiol were found to be much lower when assayed by GC–MS/MS compared to RIA in normal postmenopausal women and in breast cancer patients, either prior and during aromatase inhibitor therapy. In this study, the indirect RIA involved organic solvant extraction, celite column chromatography, use of a high affinity antibody against estradiol, and ^{125}I-labeled estradiol tracer [39]. Another work reported that estradiol levels in postmenopausal women measured by indirect RIA correlated better with GC–MS/MS values and body mass index than those obtained by direct RIA (without separation steps). Nevertheless, even with indirect RIA, estradiol levels were overestimated and less reproducible when compared with GC–MS/MS [36]. Many other studies have documented the insufficient precision and sensitivity of conventional RIA and ELISA for steroid analysis and the need to incorporate MS [38,40–43]. Thus, the Endocrine and Urology Society guidelines have highlighted the limitations of immunoassays for sex steroid hormones and have recommended the use mass spectrometric methods [44]. Since 2015, the "Journal of Clinical Endocrinology and Metabolism" requires that studies reporting on sex steroid assays published in the journal must use MS based assays [45].

It is paradoxical that at a time when the validity of RIA is under discussion and sophisticated mass spectrometric methods are

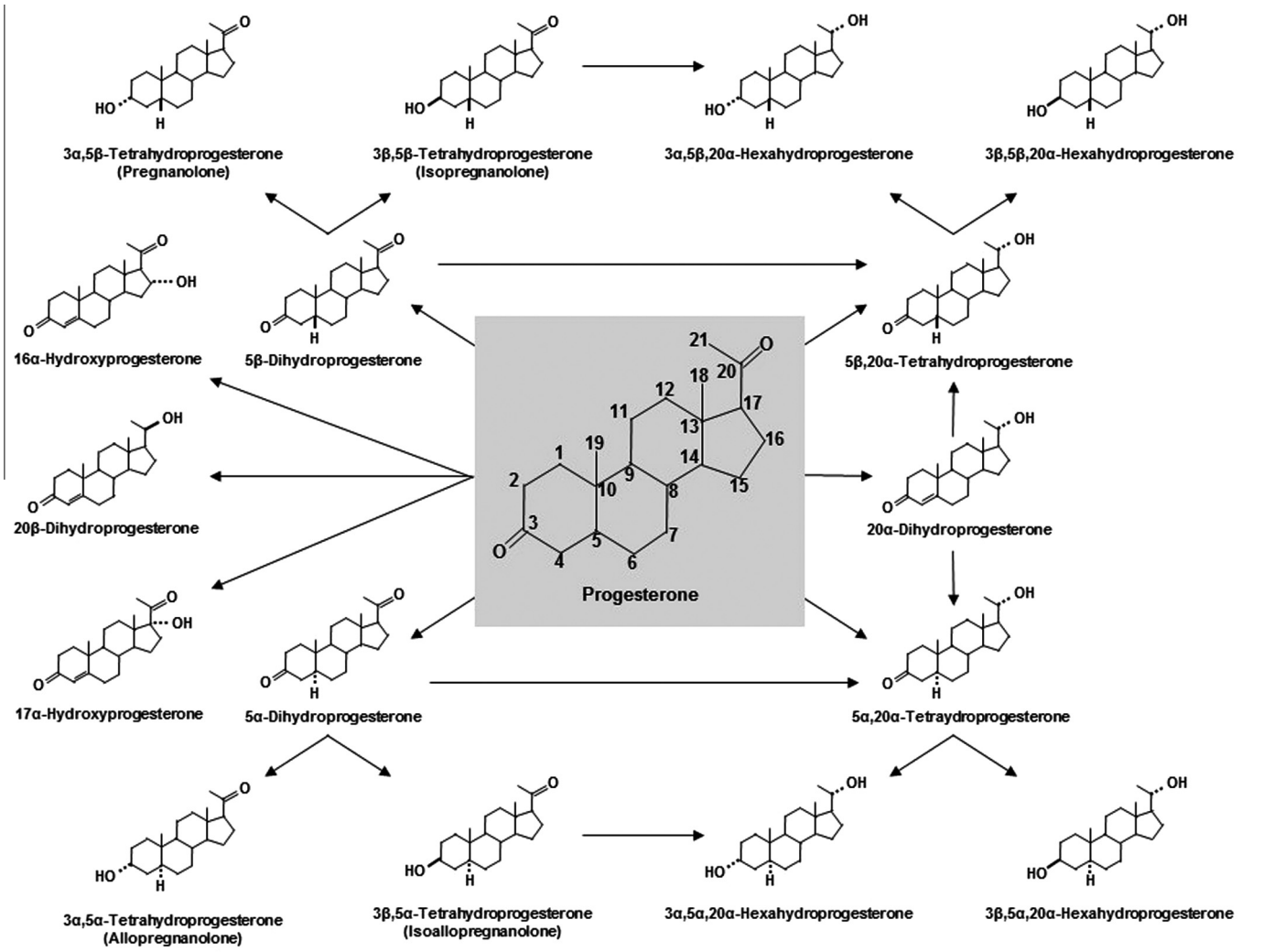

Fig. 1. Overview of 16 progesterone metabolites which have been identified by mass spectrometric methods in rat plasma and brain [62,153], or in human tissues from men and women including plasma, CSF and brain [58,60,152,165,167,244,245]. Whereas only 5α- and 20α-reduced metabolites have been detected in rat tissues, the corresponding 5β- and 20β-isomers are present in humans. Most experimental and clinical investigations of changes in the levels of progesterone metabolites have focused on 5α-dihydroprogesterone, allopregnanolone and isoallopregnanolone.

developed, or even mass spectrometers are assembled in tandem to achieve higher selectivity and sensitivity, some laboratories examine the cellular distribution and regulation of steroids in the brain by immunohistochemistry on paraformaldehyde fixed brain sections [46]. Seven years after a first report, immunohistochemistry continues to be used to study the regulation of allopregnanolone levels in specific brain cells [47–50]. In these studies, the specificity of the affinity purified allopregnanolone antibody was determined by RIA prior to immunohistochemical analysis, which is inappropriate because RIA lack sufficient specificity. Similarly, the immunostaining of estradiol on frozen brain sections has been used to document changes in estrogen levels within the hippocampus, which is not recommendable [51].

3. Analytical methods for the determination of steroid levels: mass spectrometry

Gas or liquid chromatography coupled to mass spectrometry (GC–MS or LC–MS) or tandem mass spectrometry (GC–MS/MS or LC–MS/MS) are the reference methods for accurate and robust steroid analysis. However, they are associated with high equipment and operating costs, and they require particular technical expertise, contrary to what is sometimes claimed. The access to these technologies is thus for the moment limited, although their deployment is becoming more widespread in research laboratories, and they are even making their way into clinical testing [42,52,53].

The standards for the analysis of steroid hormones by GC–MS have been developed at the Karolinska Institute in the early sixties of the last century, thus prior to the advent of the first RIA for steroids [54,55]. However, it took time before the GC–MS technology became sufficiently robust for the analysis of steroids in biological samples. Allopregnanolone and its precursors were the first steroids to be analyzed in brain tissue by GC–MS twenty years ago [56]. It is important to understand the principles behind the great specificity of GC–MS(/MS) or LC–MS(/MS) analysis of steroids. In fact, it results from the combination of the high-resolution chromatographic separation step and a specific mass spectrum. For example, steroid isomers may present a similar mass spectrum, but are separated during the chromatographic step, thus allowing their distinction and quantification. Other steroids may co-eluate during the chromatographic step, but present very distinct mass spectra. Mass spectrometric methods may thus be considered as two-dimensional with respect to chromatic separation and m/z ratio. One can of course not completely exclude the possibility that two steroids are not separated during the chromatographic step and display the same mass spectrum, but this is very unlikely. To further improve assay sensitivity and specificity, steroids need to be converted into derivatives to improve both chromatographic and spectrometric properties prior to their analysis by GC–MS(/MS) and preferably also for LC–MS(/MS). It is essential that the derivatization reactions are highly reproducible and proceed to completion for adequate accuracy and sensitivity.

There are two methodologies for the chromatographic separation of steroids prior to MS or MS/MS: GC and LC. The advantages of LC are no absolute need for steroid derivatization (although it is recommended) and the possibility to directly measure conjugated steroids. On the other hand, although requiring the derivatization of samples, GC provides higher chromatographic resolution and consequently, offers a more powerful tool for extensive steroid profiling. Indeed, the major strength of GC is its capacity to separate a large number of structurally similar steroids such as stereoisomers and enantiomers thanks to the physicochemical properties of the GC stationary phase. Thus, in addition to their exquisite specificity, methods combining chromatography with mass spectrometry offer the great advantage of accurately measuring multiple steroids in a small tissue sample, and to establish extended steroid profiles, referred to as steroid metabolomes [57–60]. The inability of measuring multiple steroids in a single sample has always be another serious limitation of conventional RIA. A large steroid profiling is indeed a prerequisite for a better understanding of changes in their levels within tissues.

However, for both GC–MS and LC–MS, mass spectrometric detection is the major challenge for assay sensitivity, reliability and specificity, which can be improved by adding a second mass spectrometer. The strategy consists in obtaining additional structural information by fragmenting in a collision cell molecular ions previously isolated from the first MS, and by analyzing selected fragment ions by the second MS. Tandem MS markedly reduces matrix interferences and background noise, and it significantly improves assay selectivity, sensitivity and precision [53,61].

Thus, mass spectrometric methods provide reference values for steroid levels and profiles in tissues and biological fluids. Recently, an extended profile of neuroactive steroids, their precursors and metabolites has been established for the adult male rat brain by GC–MS [62]. Information about the range of steroids present in the CNS is obviously a condition for understanding their biosynthesis, regulation and functions. For example, the detection of 3α,5α-tetrahydrodeoxycorticosterone, corticosterone and testosterone in the brain, but not of their direct precursors, suggests that these steroids may not be synthesized locally, but rather originate from the endocrine glands. Conversely, the presence of both the direct precursor and various metabolites of progesterone is consistent with the activity of the entire metabolic pathway in the brain [62]. Interestingly, in this GC–MS study, dehydroepiandrosterone sulfate (DHEAS), a conjugated steroid which does not exist in rodents, was detected, reminding us that mass spectrometric methods are also not free from errors. Some of the challenges facing the increasing use of mass spectrometry assays for the analysis of steroids in biological fluids and tissues have been recently discussed [59]. Here, we shall focus on the absolute requirement for carefully validated tissue sample workup.

4. The importance of sample workup prior to the analytical methods

Although mass spectrometric methods offer an unequalled tool for the analysis of steroids in biological samples, it is important to be aware that their use does not necessarily guarantee precise measures. They only become a robust technology when used properly and in combination with careful sample workup, involving multiple steps prone to errors. In this section, we address two critical points for obtaining reliable steroid measurements by MS.

First, it is essential to add suitable internal standards directly to the biological extracts for reaching high accuracy and precision for steroid quantification. Mass spectrometry using stable isotope-labeled internal standards such as deuterated or ^{13}C steroids, allows to take into account experimental losses of targeted steroids during the entire workup process before the analysis. Then, MS allows to unambiguously identify the steroids to be quantified.

It is also very important to properly validate all the purification and fractionation steps upstream of the GC–MS(/MS), LC–MS(/MS), as for RIA analysis. For example, cholesterol can be readily oxidized during the entire sample workup, both in solution in dry condition, and can give rise, under particular conditions, not only to oxysterols, but also to steroids such as pregnenolone, dehydroepiandrosterone (DHEA) and androstenediol [63]. Autoxidation constitutes a particularly serious problem for tissues rich in cholesterol such as nervous tissues. We have indeed to be aware that the myelin sheaths, which insulate axons in both white and grey matter, are composed of a high proportion of lipids (about 80% of dry

weight), and that cholesterol corresponds to more than 25–30% of the myelin lipids [64,65]. In whole rat brain, the percent of dry weight is 37 for lipids and 23 for cholesterol [66]. This very high brain cholesterol content continues to be a major cause of the erroneous detection of brain steroids.

A well-validated prepurification step of samples on reverse-phase C18 columns (also named Solid Phase Extraction, SPE) for removing cholesterol and lipids prior to steroid assay constitutes a minimal requirement. Most important, for the solubilization of cholesterol and lipids and their effective adsorption by the SPE cartridges, it is essential to resuspend steroids with pure methanol. In fact, water is frequently added to methanol in high proportion just before the SPE step. In this case, the brain extract containing the steroids is not fully dissolved, reducing the adsorption of cholesterol by the SPE cartridge. Thus, high amounts of cholesterol contaminating the steroid fraction can result in the artifactual formation of significant amounts of pregnenolone, DHEA or androstenediol [63]. The worst thing to be done is to dissolve a brain tissue extract in an aqueous medium such as methanol/water, a very commonly used and even recommended method [67].

The importance of precise analytical procedures and careful sample workup for the accurate measure of steroids is well documented by the artifactual detection of 3β-hydroxysteroid sulfates, pregnenolone sulfate (PREGS) and dehydroepiandrosterone sulfate (DHEAS) in rodent plasma and brain over decades. PREGS and DHEAS were indeed the first neurosteroids to be identified, and elevated levels were measured in the adult rat brain by RIA [68–70]. Brain PREGS attracted particular interest, as its injection at very low doses into the ventricles or directly into limbic nuclei had strong promnesic effects [71,72]. Underlying signaling mechanisms were proposed to involve modulation of gamma-aminobutyric acid type A ($GABA_A$), N-methyl-D-aspartate (NMDA) and sigma type 1 receptors [73]. Moreover, the administration of PREGS was shown to transiently correct memory deficits in aged rats. In this study, RIA analysis showed a positive correlation between the artifactual PREGS levels in hippocampus and spatial memory performance [74].

The reality of PREGS in the rodent brain was then challenged by direct analytical methods such as high performance liquid chromatography (HPLC)–electrospray ionization tandem mass spectrometry and capillary column HPLC–nanoelectrospray ionization–MS/MS, which consistently failed to detect PREGS or DHEAS [75–78]. Thus, these conjugated steroids can no longer be considered as endogenous steroids in rodents. The problem at the origin of the erroneous detection of 3β-hydroxysteroid sulfates was twofold: indirect assay procedures and contamination of the samples with cholesterol. As most antibodies used in RIA only bind to unconjugated steroids, the measure of PREGS or DHEAS requires prior solvolysis/hydrolysis to remove the sulfate group. It is the released free pregnenolone, which is then quantified by RIA. Although an antibody to PREGS had been developed, its use resulted in the detection of extremely low levels of PREGS in the rat brain by ELISA [79,80].

Likewise, 3β-hydroxysteroid sulfates can only be analyzed indirectly by GC–MS, by measuring free pregnenolone or DHEA released during the derivatization reaction (a derivatization reaction can also cleave the 3β-sulfate group, and no additional solvolysis/hydrolysis step is required). The high temperatures of the GC separation process, up to 350 °C, indeed result in the pyrolysis of conjugated steroids. Their direct analysis can only be performed by LC–MS(/MS). This explains why PREGS and DHEAS have also been initially detected in rat brain and plasma by GC–MS.

In these earlier studies, free pregnenolone and DHEA were generated during the solvolysis/hydrolysis (RIA) or derivatization (GC–MS) processes from autoxidation of cholesterol which passed through the SPE cartridges and contaminated the "sulfate fraction". This was explained by inappropriate dissolution of brain extracts in an aqueous mixture of methanol/water. Thus, both inappropriate sample preparation, with cholesterol passing through the SPE cartridges and contaminating the sulfate fraction, and the indirect assay procedure resulting in the autoxidation of the contaminating cholesterol to pregnenolone or DHEA, led to the erroneous detection of PREGS and DHEAS. This was demonstrated by a series of chromatographic and mass spectrometric studies [63,73,81]. A well-validated SPE procedure with a recycling/stepwise elution now allows to accurately analyze PREGS and DHEAS in biological samples also by GC–MS. The presence of both conjugated steroids, absent in rat and mouse brain, was thus confirmed in human brain [63].

5. Endocrine sources of CNS steroid hormones

Unconjugated steroid hormones, which are produced by the gonads and adrenal glands, easily pass the blood–brain–barrier (BBB) and the blood-spinal cord barrier by transmembrane diffusion [82,83]. They also rapidly diffuse throughout the nervous tissues because of their low molecular weight (~300 Da) and their lipid solubility. Thus, in response to acute stress, the adrenal glands secrete corticosterone in rodents or cortisol in humans into the circulation, which then rapidly enter the brain. As an example, in response to swim stress or a novel environment, levels of free corticosterone measured by *in vivo* microdialysis within the male rat hippocampus peaked after 30–45 min. This was about 20 min later when compared to increased levels in plasma. Like for the endogenous hormone, hippocampal levels of corticosterone were increased within only 15 min after its subcutaneous injection [84].

However, adrenal and gonadal steroid hormones differ with respect to their ability to cross the BBB. Their permeability is indeed inversely related to the number of hydrogen bonds that they form in aqueous solution. For this reason, progesterone, testosterone and estradiol penetrate more easily the CNS than corticosterone or cortisol. Moreover, the binding of steroid hormones to plasma proteins has an influence on their brain penetration kinetics [83,85]. Another factor interfering with the access of steroid hormones to the CNS is the presence of multidrug-resistance transporters (MDR) at the level of the BBB. These ATP-binding cassette glycoproteins carry a wide range of xenobiotics, including steroids, out of the brain. The use of mice deficient for MDR has demonstrated that these proteins provide an endogenous barrier system for corticosterone, cortisol, and in particular for synthetic steroid compounds such as dexamethasone. In contrast, intracerebral levels of progesterone were only slightly affected by the absence or presence of MDR [86,87]. In addition, the penetration of steroids into the brain may be affected by environmental stimuli and physiopathological conditions. Thus, the passage of progesterone and estradiol into the CNS of female sheep, a seasonal breeder, is influenced by the photoperiod. Cerebrospinal fluid (CSF) levels of progesterone were higher during short days, whereas CSF levels of estradiol were higher during long days, and these changes did not reflect plasma levels of these hormones [88,89].

6. Production of neurosteroids in the CNS

In addition to the gonads and adrenal glands, steroids are also synthesized *de novo* from cholesterol and metabolized within the CNS. To refer to their site of synthesis, steroids produced within the nervous system have been named "neurosteroids" [90,91]. For example, progesterone and allopregnanolone are not only synthesized by the ovaries and adrenal glands, but also by neurons and glial cells within the nervous system. In addition, steroids produced by the endocrine glands, can be converted to neuroactive

metabolites within the brain. The term "neuroactive steroids" has been initially coined to designate those steroids which modulate neurotransmitter receptors and thus rapidly alter the activity and excitability of neurons [92]. However, this concept may be extended to all steroids that directly act on neural cells, either via membrane or nuclear signaling mechanisms.

It is not always evident whether changes in CNS steroid levels reflect local changes in neurosteroid synthesis or responses of the endocrine glands to specific stimuli or both. So far, no conditional knockouts of neurosteroid biosynthetic pathways have been generated, and studies are generally limited to: (i) the expression and activity of steroidogenic enzymes; (ii) the comparison of steroid concentrations between brain and plasma; (iii) the measure of steroids remaining in CNS tissue after removal of the peripheral endocrine glands. Thus, after castration and adrenalectomy, significant levels of pregnenolone and progesterone remain present in CNS and peripheral nerves but not in plasma, these results being consistent with their local synthesis in nervous tissues [93–95]. Comparing steroid levels, it has to be pointed out that they are generally expressed for plasma as nanogram per milliliter (ng/ml) and for brain tissue as ng/gram (gr) or for both in nanomolar concentrations. These different units are equivalent as the densities of blood plasma or serum and brain tissue are very similar (~1 g/ml) [96].

The authenticity of pregnenolone, progesterone, 5α-DHP and allopregnanolone in various regions of the male rat brain, thus of all components of the biosynthetic pathway of allopregnanolone, has then been confirmed by GC–MS preceded by a HPLC pre-separation step [56]. Importantly, although levels of all four steroids were significantly reduced in the brain after removal of their peripheral source by adrenalectomy, significant amounts remained present, suggesting that both the endocrine glands and local synthesis may contribute to the pool of steroids present in the brain. The formation of allopregnanolone from progesterone in the brain was demonstrated by treatment of the animals with the selective 5α-reductase inhibitor SKF105111. As in many other studies of this time, indirect assay procedures led to the erroneous detection of PREGS [56].

Although the persistence of steroids in nervous tissues after castration and adrenalectomy is considered to reflect their local synthesis, little information is available concerning the absorption, retention and turnover of different steroids in the CNS. The uptake by the brain of systemically injected tritium ([^{3}H])-labeled steroids has been extensively investigated in the seventies, with the aim to identify their selective retention on receptor sites [97,98]. However, these studies do not provide information about the different brain pools of steroids, either available for metabolic and signaling processes or trapped within lipid-rich structures such as white matter. The fact that peripherally administered [^{3}H]-labeled pregnenolone is quickly taken up and again rapidly released from the brain is consistent with the hypothesis that the persistence of steroids after gonadectomy and adrenalectomy may not be due to their retention and accumulation in tissues, but may rather reflect their local synthesis. Thus, the concentration of [^{3}H]pregnenolone was several-fold higher in brain than in plasma of rats between 1 and 7 h after its subcutaneous injection, but only trace amounts could be detected in brain after 20 h [69]. Interestingly, the retention time of [^{3}H] progesterone in rat brain was even much shorter, only 30 min [99]. This can be explained by different pharmacokinetics and metabolism. Thus, pregnenolone may conjugate with free fatty acid esters and may be trapped more easily in lipid-rich structures.

Additional support for the local synthesis of neurosteroids was provided by a study showing that inhibition of the metabolic conversion of pregnenolone to progesterone in castrated and adrenalectomized rats resulted in a significant increase in brain pregnenolone levels. In this experiment, trilostane, a competitive inhibitor of the 3β-hydroxysteroid dehydrogenases (3β-HSD), which convert pregnenolone to progesterone, was subcutaneously injected twice daily for 7 days. Brains were then sampled and processed for steroid analysis 12 h after the last injection [100]. Other strong evidence for the *de novo* synthesis of neurosteroids in the brain came from a series of studies using agonist ligands of the translocase 18 kDa (TSPO), the former peripheral benzodiazepine receptor (PBR) [94,101–104]. As mentioned above, the conversion of cholesterol to pregnenolone by cytochrome P450scc takes place at the inner membrane of steroidogenic mitochondria. However, cholesterol cannot enter the mitochondria by simple diffusion because of its hydrophobicity, but requires carrier proteins, including TSPO and the steroidogenic acute regulatory protein (StAR) (for the actual controversy concerning these two carriers, see: [105–108]). The administration of TSPO agonist ligands has previously been shown to stimulate the synthesis of steroids in CNS and endocrine glands [8,109,110].

7. Changes in brain steroid levels in response to stress

In a seminal study, acute swim stress was shown to increase markedly, but transiently, both progesterone and allopregnanolone levels in plasma and brain of young adult male rats (see Fig. 1 for the metabolic pathway of allopregnanolone formation). Steroids were extracted from homogenized brain tissue in 50% aqueous methanol. Allopregnanolone was measured by RIA after separation of steroids cross-reacting with the antibody by HPLC. The other steroids were analyzed by using RIA kits [111]. Several findings of this study deserve our attention. In plasma, progesterone levels markedly raised after a 10 min stress, whereas plasma levels of allopregnanolone only modestly increased. However, both steroids reached similar high levels in brain. The parallel increase in plasma and brain progesterone in response to stress may reflect a stimulation of progesterone secretion by the adrenal glands. On the other hand, adrenal progesterone is actively converted to allopregnanolone within the brain. In fact, levels of both progesterone and allopregnanolone were very low in plasma and brain after adrenalectomy. Taken together, these results point to a major contribution of the adrenal glands to the elevation of brain progesterone and allopregnanolone levels in response to acute stress. Nevertheless, higher levels of progesterone and allopregnanolone measured in the brain of unstressed males when compared to plasma levels, as well as the persistence of measurable levels of allopregnanolone in the brain of stressed males after adrenalectomy, point to an additional brain synthesis. Brain levels of tetrahydrodeoxycorticosterone (THDOC) were also increased in response to acute stress, but in contrast to allopregnanolone, this neuroactive steroid could no longer be detected after adrenalectomy [111].

That the adrenal glands are a major source of brain allopregnanolone has also been shown after foot shock. After this type of acute stress, the transient and rapid increase in allopregnanolone in the male rat brain was indeed prevented by removal of the adrenal glands [112]. In this study, levels of allopregnanolone were measured by RIA after HPLC separation of steroids, as in the study discussed above, and the allopregnanolone content of random samples was verified by GC–MS [112]. The same authors also showed that the increase in brain allopregnanolone after an acute stress caused by CO_2 inhalation, a paradigm known to induce panic attacks in humans, could be prevented in male rats by removal of the adrenal glands [113].

The increase in allopregnanolone within the male rat brain and plasma in response to acute swim stress was later confirmed by GC–MS analysis [114]. Interestingly, levels of the precursor pregnenolone were also found to be increased in brain in response to stress, a change not reflected in plasma levels, pointing to a

possible stimulation of *de novo* synthesis [114]. When measured by LC–MS/MS (LC coupled with electrospray ionization, ESI), levels of brain allopregnanolone after immobilization stress (1.74 ng/g) agreed well with those measured by GC–MS after swim stress (1.3 ng/g) [115]. However, allopregnanolone levels determined by the mass spectrometric methods were much lower than those measured by RIA after HPLC (about 12–50 ng/g) [111,113,115]. This may reflect the much greater selectivity of GC–MS and LC–MS/MS when compared to RIA, even with the use of a preceding HPLC separation step.

Whatever the sources of allopregnanolone and THDOC, the rapid increase in their brain levels in response to stress may be part of adaptative responses. By restoring the GABAergic tone after stress, they may moderate the activation of the hypothalamo-pituitary–adrenal (HPA) axis, the major neuroendocrine stress response system, and exert anxiolytic, analgesic and anticonvulsant actions [113,116–118]. Systemic treatment of adrenalectomized rats with allopregnanolone indeed reduced expression of corticotropin-releasing hormone (CRH) within the hypothalamic periventricular nuclei. On the other hand, treatment of rats with the 5α-reductase inhibitor finasteride increased their ACTH and corticosterone responses to acute stress [118,119]. Allopregnanolone and THDOC may thus be considered as endogenous stress-protective hormones. It is interesting to note that gonadal steroid hormones can also modulate in a sex-specific manner stress reactivity and neuroendocrine responses to stress [120]. Thus, testosterone blunts the activity of the HPA axis in males via its conversion to 3β,5α-androstanediol [121–123].

In contrast to the rapid and transient increase in brain steroids in response to acute stress, exposure to chronic stressful situations such as prolonged social isolation resulted in significantly decreased levels of brain steroids, including pregnenolone, progesterone, allopregnanolone and THDOC, and in decreased 5α-reductase activity [124,125]. In male and females rats socially isolated for 1 month immediately after weaning, both plasma and brain levels of allopregnanolone were markedly decreased. A reduced activity of the HPA axis was proposed to be responsible for the chronic decrease in allopregnanolone [126]. In these chronic stress studies, allopregnanolone was measured by RIA after HPLC pre-purification, reporting absolute levels higher than those determined by spectrometric methods.

As neuroactive steroids play a central role in the physiological adaptation to stress, their inadapted responses resulting from chronic exposure to stress may contribute to the development of stress-related disorders, such as anxiety disorders and depression. Not only severe chronic stress, but also the stressful daily life events may progressively contribute to a chronic burden, qualified as "allostatic load", with negative consequences for health [127]. Surveillance of neuroactive steroid profiles in plasma or CSF and their possible normalization may thus offer interesting perspectives for the prevention and treatment of stress-related disorders.

8. Changes in brain steroid levels in post-traumatic stress disorder

In premenopausal women with post-traumatic stress disorder (PTSD), an emotional disorder resulting from traumatic life events, GC–MS analysis revealed decreased CSF levels of allopregnanolone. Moreover, the ratio of allopregnanolone/DHEA correlated negatively with PTSD symptoms and with depression scores. CSF levels of allopregnanolone were lowest in patients with PTSD and comorbid depression [128]. PTSD is a complex disorder affecting many regulatory systems, including alterations of the HPA axis [129–131]. Interestingly, alterations of the $GABA_A$ neurotransmitter system associated with decreased allopregnanolone levels have also been reported [128,132]. Again, socially isolated mice are used to explore therapeutic options for the pharmacological treatment of PTSD. Thus, treatment of socially isolated mice with ganaxolone, a synthetic analog of allopregnanolone, has been recently shown to improve behavioral deficits [132].

There exists another potential therapeutic strategy for the treatment of mood disturbances: the stimulation of neurosteroid synthesis in the brain by TSPO agonist ligands [8,133]. The increase in brain steroid levels in rats deprived of their steroidogenic endocrine glands in response to TSPO ligand administration is a strong argument in favor of the reality of neurosteroid synthesis within the CNS. Proof of principle for the efficient use of TSPO ligands as therapeutic agents for the treatment of psychiatric disorders have been provided by experimental studies and a recent clinical trial [8,134].

By binding to TSPO, the benzoxazine etifoxine exerts part of its anxiolytic effects by stimulating the synthesis of the positive $GABA_A$ receptor modulator allopregnanolone [94]. Evidence for a clinical anxiolytic effect of etifoxine has been provided by a double-blind controlled study of patients suffering from adjustment disorders with anxiety [135]. However, as etifoxine directly binds to both $GABA_A$ receptors and TSPO, the extent to which its anxiolytic effects were related to the stimulation of allopregnanolone synthesis was not clear. More recently, the selective TSPO ligand XBD173 has been demonstrated to enhance GABAergic neurotransmission via the stimulation of allopregnanolone synthesis when it was measured by GC–MS. XBD173 also blunted pharmacologically induced panic attacks in rats in the absence of sedation [136]. Most importantly, XBD173 displayed antipanic and anxiolytic efficacy in humans using an experimental anxiety paradigm that involves challenge with cholecystokinin tetrapeptide (CCK4) [136].

9. Changes in brain steroid levels in depression

Stress is a normal physiological response to environmental challenges, which mobilizes energy and improves performance. However, chronic stress has adverse effects on the brain and may lead to anxiety disorders, depression and even to neurodegenerative events [137–139]. An impaired regulation of the HPA axis has been associated with these stress-related disorders [140]. The adaptative functions of brain steroids are thus likely to play an important role. The possibility that a downregulation of neurosteroid biosynthesis may contribute to the development of anxiety and depressive disorders has attracted the attention of researchers over the past two decades.

A first experimental result consistent with this concept was the observation that selective serotonin reuptake inhibitors (SSRI), which are commonly prescribed antidepressants, stimulate brain neurosteroid synthesis. Thus, intraperitoneal administration of fluoxetine or paroxetine to adrenalectomized and castrated male rats increased in a dose-dependent manner the content of allopregnanolone in brain in a region-specific manner, but not in plasma. Steroids were quantified by GC–MS after HPLC separation of the different steroid fractions. Interestingly, an additional purification step on silica gel thin layer chromatography (TLC) was used to remove contaminating cholesterol from the fraction containing 5α-DHP [141]. In the same study, it was shown that the addition of 5α-DHP to rat brain slices preincubated with fluoxetine elicited a rapid accumulation of allopregnanolone, much greater than in slices preincubated with vehicle. This result suggests that fluoxetine may promote the conversion of 5α-DHP to allopregnanolone. Studies *in vitro* using recombinant rat enzymes showed that fluoxetine, paroxetine, or sertraline indeed stimulated the formation of allopregnanolone by increasing the affinity of the 3α-hydroxysteroid dehydrogenase (3α-HSD) for 5α-DHP. However, the

antidepressants did not activate the 5α-reductase type 1, which is the main isoform of the enzyme in the rodent brain [142].

By potentiating $GABA_A$ receptor functions, allopregnanolone may alleviate anxiety and dysphoria associated with depression. CSF levels of allopregnanolone observed by GC–MS were indeed decreased by 60% in patients with major unipolar depression. However, treatment with fluoxetine normalized the CSF content of allopregnanolone. Moreover, a significant correlation existed between symptomatology improvement and the increase in CSF allopregnanolone after fluoxetine treatment [143]. In contrast, the CSF content of pregnenolone and progesterone remained unaltered after treatment. Results of this study suggest that a normalization of allopregnanolone levels in depressed patients may contribute to the improvement of depressive symptoms. As fluoxetine and related antidepressant drugs normalize levels of allopregnanolone and facilitate $GABA_A$ receptor neurotransmission, they have been qualified as "selective brain steroidogenic stimulants" [144]. In support of the hypothesis that CSF levels of allopregnanolone may indeed reflect decreased brain allopregnanolone synthesis, a recent study reported that mRNA levels of the 5α-reductase are significantly decreased in prefrontal cortex of depressed patients [145].

Interestingly, decreased levels of allopregnanolone and increased levels of iso-allopregnanolone (which inhibits the activity of $GABA_A$ receptors), were measured by GC–MS also in plasma of patients with major depression. This disequilibrium of neuroactive plasma steroids could also be corrected by treatment with different antidepressants [146]. In contrast to allopregnanolone, plasma levels of 5α-dihydrodeoxycorticosterone (5α-DHDOC) and 3α,5α-THDOC, another potent positive modulator of $GABA_A$ receptors, were increased in depressed patients. Plasma 3α,5α-THDOC was either unaffected or decreased in response to antidepressant treatment [147,148]. Furthermore, like the SSRI, the tetracyclic antidepressant Mirtazapine, which increases noradrenergic and serotonergic neurotransmission, affected plasma levels of neuroactive steroids analyzed by GC–MS. Mirtazapine was shown to increase levels of allopregnanolone by inhibiting its conversion back to 5α-DHP [149].

It is remarkable that changes in neurosteroid levels during depressive episodes and the effects of antidepressant treatments have been much studied in patients. A limitation is the difficulty to appreciate whether plasma or CSF levels of neuroactive steroids reflect their brain concentrations. A study using GC–MS with careful sample workup has demonstrated that post-mortem CSF levels of pregnenolone and DHEA positively correlated with those measured in temporal cortex [150]. However, concentrations of steroids in CSF measured by GC–MS are in general much lower than in the circulation [151]. It has been proposed that neuroactive steroid profiles in plasma may be as good biomarkers for CNS disorders than those measured in CSF [152]. This assumption is consistent with the similar effects of antidepressants on neuroactive steroid levels in CSF and plasma.

A recent study has compared neuroactive steroid profiles in plasma, CSF and nervous tissues, including cerebral cortex, cerebellum, hippocampus, spinal cord and sciatic nerve, of male and female rats. Ten steroids were analyzed by LC–MS/MS within individual samples after the addition of deuterated or ^{13}C labeled internal standards for recovery calculations, followed by solvant extraction: pregnenolone, progesterone, 5α-dihydroprogesterone, allopregnanolone, isopregnanolone, DHEA, testosterone, 5α-dihydrotestosterone, 3α-androstanediol and estradiol. Although CSF levels of steroids not fully reflected their nervous tissue levels, positive correlations were found for pregnenolone, progesterone, testosterone, 5α-dihydrotestosterone and estradiol [153].

Like for many psychiatric disorders, there are no satisfactory animal models of depression. Only particular phenotypes of this complex disorder can be reproduced in rodents, including anxiety, loss in motivation and energy, behavioral despair, cognitive disturbance, alterations of circadian rhythms and neuromorphological changes [154]. Because of the association between stress and mood disorders, many stress-based models of depression have been developed; among them, the Porsolt forced swim test, chronic mild stress and social isolation [154,155]. The fact that behavioral traits in these models are affected by treatment with antidepressant drugs strongly suggests that they might be relevant to depression.

We have already discussed above the stimulating effect of acute stress on brain steroid levels and their decrease in response to chronic stress. Importantly, levels of neuroactive steroids are not only decreased in chronically stressed rats, but the increase in brain allopregnanolone normally observed in response to acute stress was also lost [156]. A large number of experimental studies have used rodents exposed to a protracted period (3–4 weeks) of social isolation stress as a model to examine behavioral changes associated with depression and other emotional disorders. These studies have already been extensively reviewed [144]. In short, they show that in socially isolated mice, behavioral abnormalities are associated with a marked decrease in brain allopregnanolone content and synthesis [157]. Importantly, treatment with allopregnanolone improved the behavioral deficits and the administration of fluoxetine allowed normalizing brain allopregnanolone levels. It should also be pointed out that many of these studies have been carried out in the laboratory of Erminio Costa and his collaborators, who were the pioneers of neurosteroid analysis by GC–MS in nervous tissues [56].

10. Changes in brain steroid levels in response to neurodegeneration: Alzheimer's disease

As in response to stress, brain steroid levels are affected during neurodegenerative diseases. There is now strong evidence that steroid hormones and neurosteroids could play an important role in a variety of degenerative diseases of the CNS, ranging from Alzheimer's disease (AD) to multiple sclerosis (MS) [158]. Some changes in brain steroid levels could result from or may even contribute to the degenerative processes, whereas others may be part of endogenous protective responses and rescue programs. Here, we shall focus on AD and MS, two degenerative conditions for which beneficial effects of steroids or TSPO ligands have been demonstrated in experimental models.

In AD, the HPA-axis is only moderately activated and there is currently no strong argument for a major role of cortisol in the disease process [159,160]. However, as in stress disorders and depression, there is evidence for reduced allopregnanolone levels in brain, CSF and plasma. These results gain their pathophysiological significance from the experimental work on the protective and neuroregenerative actions of allopregnanolone [3,161]. Thus, repeated measures revealed reduced serum levels of allopregnanolone in patients with AD when compared to control subjects. In this study, allopregnanolone was measured by RIA in pre-purified serum on SPE cartridges [162]. In a second study, among seven steroids measured by RIA following Celite column chromatography, only allopregnanolone was found to be significantly decreased in plasma of AD and demented patients when compared to controls [163]. In both studies, as for a large number of published allopregnanolone RIA studies, a polyclonal antiserum provided by Dr. Robert H. Purdy, has been used. It was raised in sheep against allopregnanolone coupled to bovine serum albumin and requires chromatographic separation of allopregnanolone as it cross-reacts with a series of steroids, in particular 5α-reduced metabolites of corticosterone and deoxycorticosterone [111,163,164]. It is important to draw attention to the fact that the necessary chromatographic step has been omitted in a series of other studies, and that this

antibody has been used in the above mentioned immunohistochemical detection of allopregnanolone on rat brain sections.

Reduced levels of allopregnanolone were also measured in the temporal cortex of AD patients by GC–MS preceded by HPLC. Moreover, they were found to inversely correlate with neuropathological disease stages [165,166]. Results of another study using GC–MS analysis also pointed to decreased levels of allopregnanolone within different brain regions of AD patients without reaching statistical significance because of great sample variability [167].

At first glace, these findings may be interpreted in favor of an alteration of the biosynthetic pathway of allopregnanolone in AD brains. However, a recent study of enzyme mRNA expression by quantitative RT-PCR in the brains of AD patients at different disease stages now sheds a new light on these results. Thus, levels of mRNA coding for the 3α-HSD (AKR1C2, enzyme which converts 5α-DHP to allopregnanolone), as well as those coding for other enzymes and cholesterol transporters involved in steroid biosynthetic pathways, were found to be increased at early and later stages of AD [158,168]. These results rather point an increased synthesis of allopregnanolone in AD. Moreover, as increases in mRNA levels already start at early stages of AD with mild cognitive impairment, they may reflect an attempt to increase the synthesis of neurosteroids with neuroprotective effects and represent an early protective and compensatory mechanism. Thus, there is not necessarily a correspondence between enzyme expression and steroid levels.

Among those neuroactive brain steroids that may be compensatory to the undergoing neurodegenerative processes in AD, DHEA deserves our particular attention because of its trophic and protective actions [169,170]. Moreover, there is evidence that DHEA may protect against amyloid beta toxicity [171,172]. Levels of DHEA were found to be significantly increased in hypothalamus, hippocampus and frontal cortex of post-mortem AD brains when compared to age-matched controls. DHEA levels in CSF were also significantly higher in AD, whereas plasma levels of DHEA did not differ between AD and controls [173]. In this study, steroid levels were determined by RIA after HPLC separation, and the identity of DHEA was confirmed by GC–MS. Interestingly, no cytochrome P450 17α-hydroxylase (P450c17) could be detected by immunohistochemistry in hippocampus, which however contained the highest levels of DHEA. The authors indeed provided evidence for an alternative DHEA biosynthetic pathway in hippocampus stimulated by ferrous sulfate ($FeSO_4$) [173]. Increased CSF levels of DHEA were also measured in living AD patients, again by RIA after HPLC separation and GC–MS verification. However, although CSF levels of DHEA significantly differed between AD patients and control subjects, there was a large overlap between individual measures [174]. In a subsequent study, post-mortem CSF levels of DHEA and its precursor pregnenolone analyzed by GC–MS positively correlated with those measured in temporal cortex and with AD disease stage [150]. In contrast, another study reported reduced plasma levels of DHEA in AD when compared to non-demented controls [162]. The different results may reflect differences in samples or in analytical procedures. DHEA levels were measured by RIA after a prepurification step on SPE cartridges.

In support of the concept that increased neurosteroid synthesis may be part of natural protective or rescue programs is the induction of aromatase expression in astrocytes at late stages of AD [158,168]. The aromatase has indeed been qualified as a neuroprotective enzyme, as its inhibition by pharmacological or genetic tools increases the vulnerability of the brain to injury [5,175]. Increased aromatase immunostaining has also been detected in neurons of the Nucleus Basalis of Meynert of AD patients, which is strongly affected by the disease [176]. However, levels of estradiol, resulting from the aromatization of testosterone, have been found unchanged in the prefrontal cortex of AD patients. But in this study, estradiol was measured by RIA following extraction with ethyl acetate: hexane and separation by Celite column partition chromatography [177]. In men with AD, brain levels of testosterone were decreased, but not those of estradiol [178].

A currently used animal model of AD is the 3xTgAD mouse harboring transgenes of mutant presenilin 1 (PS1 with mutation M146), amyloid precursor protein (APP with the Swedish mutation), and Tau (with mutation P301L) [179]. Steroid profiling by LC–MS/MS in male 3xTgAD mice compared to wild-type mice at the ages of 7 months and 24 months revealed changes in neuroactive steroid levels within the limbic brain [180]. Consistent with therapeutic perspectives for TSPO ligands, the selective agonist Ro5-4864 attenuated and even reversed neuropathological and behavioral impairment in the 3xTgAD mice while significantly increasing brain levels of progesterone and testosterone, also analyzed by LC–MS/MS [181].

11. Changes in brain steroid levels in response to myelin degeneration: multiple sclerosis

When discussing a possible role of CNS steroids in endogenous protective and rescue mechanisms, attention should not be exclusively focused on neurons and synaptic connections, but also on myelin. The lipid-rich myelin sheaths, which insulate large diameter axons and are necessary for the rapid saltatory conduction of nerve impulses, are required for the proper functioning of neuronal circuits. The importance of myelin is documented by the neurological consequences of demyelinating diseases such as multiple sclerosis [182,183].

Within the CNS, the myelin sheaths are formed by a particular type of glial cells, the oligodendrocytes, which extend their processes and envelop stretches of axons with compact myelin [184]. Although myelin is damaged by autoimmune reactions in MS, oligodendrocytes are also very sensitive to changes in their microenvironment, in particular to oxidative stress, glutamate excitotoxicity, inflammation and oxygen/glucose deprivation [185,186]. Important for our purpose is that myelin and oligodendrocytes can regenerate, even in the adult CNS, thus contrasting with the limited capacity of neuronal regeneration. The remyelination of demyelinated axons requires the generation of new oligodendrocytes from oligodendrocyte precursor cells (OPC), which are abundant and largely distributed throughout the adult brain and spinal cord [187,188]. Thus, in the case of myelin, CNS steroids may not only be part of protective and rescue programs, but also stimulate endogenous regenerative mechanisms and repair [14,189,190].

Progestogens, estrogens and androgens have indeed been shown to promote myelin regeneration in the CNS by stimulating the proliferation and differentiation of OPC and by promoting the synthesis of new myelin [9,191–195]. In addition to their regenerative actions, CNS steroids exert protective effects on mature oligodendrocytes [196–198]. Remyelinating, neuroprotective and anti-inflammatory actions effects of androgens, estrogens and progestogens have also been demonstrated in experimental autoimmune encephalomyelitis (EAE), a widely used animal model of MS [199–203]. EAE can be induced in rats or mice by direct immunization with myelin proteins or peptides (active EAE, the most commonly used model) or by the transfer of autoreactive T cells taken from animals which have been immunized against a particular myelin antigen (passive EAE) [204].

The effects of glucocorticoids appear complex, as they have been shown to delay the maturation of OPC, but also to promote remyelination after toxin-induced demyelination and to exert protective effects on oligodendrocytes [205–208]. Glucocorticoid

synthesis is increased in MS, but activation of the HPA axis is dependent on the type of MS, relapsing-remitting or progressive [158,209]. CSF levels of cortisol, measured by RIA, were elevated in patients with severe MS and highly correlated with serum levels [210]. However, low CSF and normal plasma levels of cortisol were found in patients with relapsing remitting MS. The lowered cortisol levels in these patients were related to the elevated expression of the 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2), enzyme which inactivates cortisol by catalyzing its conversion to cortisone [211]. However, these results have to be interpreted with caution, as cortisol concentrations were determined by "luminescent immunoassay according to the manufacturer's instructions".

Recently, neuroactive steroid levels have been analyzed by LC–MS/MS in both CSF and plasma of adult male patients affected by relapsing-remitting MS [212]. In CSF, a decrease in the progesterone metabolites 5α-DHP and allopregnanolone was associated with an increase in levels of pregnenolone and isoallopregnanolone. Furthermore, 5α-dihydrotestosterone and estradiol levels were decreased. In plasma, 5α-DHP and allopregnanolone levels were also decreased, whereas concentrations of pregnenolone, progesterone, isoallopregnanolone and 5α-dihydrotestosterone were increased [212].

That changes in neurosteroid levels may involve active regulation of enzyme expression has been recently shown by a noteworthy study on micro-RNAs in cerebral white matter of MS patients of both sexes collected at autopsy [213]. Micro-RNAs are small RNA molecules involved in the regulation of gene expression through translational silencing or the degradation of RNA [214]. High-throughput microarray micro-RNA profiling showed that 3 neurosteroid synthesis enzyme-specific micro-RNAs were induced in MS brains. The neurosteroidogenic enzymes specifically targeted by these micro-RNAs were those involved in the conversion of 5α-DHP to allopregnanolone (3α-HSD or AKR1C2) and of progesterone to 20α-dihydroprogesterone (20α-HSD or AKR1C1). Consistently, levels of allopregnanolone measured by GC–MS were found decreased in the white matter of MS patients. Notably, two of these micro-RNAs were also induced in mice affected by EAE, accompanied by a diminished expression of allopregnanolone biosynthetic enzymes. Treatment of EAE mice with allopregnanolone reduced neuroinflammation, myelin and axonal injury and behavioral deficits [213,215].

Gender-specific changes in the expression of neurosteroidogenic enzymes within MS lesions have been reported recently. By combining quantitative RT-PCR and immunohistochemistry, it was shown that the aromatase was locally upregulated in MS lesions of males, together with estrogen receptor-β (ERβ). Instead, in MS lesions of females, the 3β-hydroxysteroid dehydrogenase (3β-HSD), enzyme that converts pregnenolone to progesterone, was upregulated, together with PR [216].

The demonstration of an important role of neurosteroids in MS offers therapeutic promises for protective and regenerative effects of TSPO ligands. Thus, the positive modulation of TSPO activity with etifoxine has been shown to ameliorate disease symptoms and neuropathology in EAE mice [217]. Etifoxine attenuated EAE severity when administered before the development of clinical signs and improved symptomatic recovery when administered at the peak of the disease. Importantly, recovery was associated with diminished neuroinflammation and increased oligodendrocyte regeneration [217].

12. Changes in CNS steroid levels in response to traumatic injury

Neuroprotective effects of progesterone, estradiol and testosterone have been demonstrated in a variety of CNS injury models, including models of traumatic brain injury (TBI), spinal cord injury (SCI), ischemic stroke (middle cerebral artery occlusion, MCAO) and excitotoxic brain damage. They have already been extensively reviewed [1–6,218–224]. To provide further support for our hypothesis that changes in endogenous CNS steroid levels may reflect adaptative processes, it is important to also examine how they vary in response to injury. Here, our investigation will be limited to traumatic and to ischemic damage, where the protective effects of estradiol and progesterone have been well documented. Another important question to be addressed is to what extent changes in circulating hormones may reflect changes in CNS steroids.

After fluid-percussion injury, the conversion of intravenously administered PREGS to progesterone, 5α-DHP and allopregnanolone was strongly increased in the brain and spinal cord [225]. More recently, profiling of neuroactive steroids has been performed in plasma and brain of young adult female mice in response to weight-drop TBI. Steroids were analyzed by LC–MS/MS, but samples were resuspended in methanol/H_2O (10/90, v/v) prior to the SPE purification step, which may have prevented the solubilization and efficient adsorption of lipids and cholesterol by SPE cartridges. So, this protocol may explain the detection of DHEA in the samples, which is normally not present in the mouse or rat brain [81]. Interestingly, brain levels of progesterone, allopregnanolone, isoallopregnanolone, testosterone and 17β-estradiol were found to be decreased 24 h after TBI, and they remained low during the following 2 weeks [226]. In plasma, levels of progesterone were also markedly decreased between 24 h and 2 weeks after TBI. Thus, the decrease in brain progesterone may result from a reduction in plasma levels. Pituitary dysfunction and alterations of the hypothalamo-pituitary gonadal and hypothalamo-pituitary–adrenal axes are indeed a well-known consequence of TBI [227,228]. Noteworthy, brain progesterone levels, but not its plasma levels, showed a positive correlation with neurological recovery, consistent with a protective role [226].

Steroids have also been analyzed by GC–MS after focal injury of the male rat prefrontal cortex. Six hours after TBI, brain levels of pregnenolone, progesterone and 5α-DHP were increased and those of testosterone decreased within, adjacent or distal to the lesion site. Plasma levels of corticosterone were also significantly increased, reflecting adrenal gland activation by TBI. The difference in brain steroid levels were no longer observed 24 h after TBI, except for pregnenolone [229].

CSF levels of estradiol and testosterone have been analyzed daily during 6 days in 110 men and women after TBI and compared with healthy controls. TBI subjects displayed lower CSF estradiol, while CSF testosterone initially high in men, declined over time. Elevated mean estradiol/testosterone ratio was associated with lower mortality and better outcomes [230]. A major limitation of the study was that steroids were analyzed by using an RIA kit according to manufacturer's instructions.

Temporal profiles of serum gonadal and adrenal hormones were analyzed in a cohort of patients with severe TBI during the acute phases of their injury and compared to those of healthy controls. One objective of the study was to test whether steroid hormones may be used as biomarkers for predicting TBI outcome. Particularly interesting was the strong increase in plasma progesterone levels shortly after TBI in men and returning to control levels within 1 day. This transient increase in plasma progesterone most likely reflected stimulation of the adrenal gland and may be neuroprotective [231]. In contrast to progesterone, levels of cortisol remained elevated in both sexes [232].

Levels of pregnenolone and progesterone measured by GC–MS were also strongly upregulated in different parts of the male rat spinal cord after its transection, even after removal of the steroidogenic glands by combined castration and adrenalectomy. As expected, in the absence of the steroidogenic glands, no changes

in plasma steroid levels were observed. These observations thus provide strong evidence for an increase in neurosteroid synthesis in response to injury. Particularly interesting was the time-course of the increases in spinal cord pregnenolone and progesterone in the absence of the testes and adrenal glands: while pregnenolone levels were strongly upregulated 24 h after injury, progesterone levels were only increased after 75 h, and concomitantly with a marked decrease in pregnenolone to below control levels. An interpretation of these results is that the pregnenolone biosynthetic machinery may be first activated in response to injury, followed by the activation of progesterone synthesis and consumption of the pregnenolone substrate [233].

These results strongly suggest that the upregulation of pregnenolone and progesterone synthesis within the spinal cord in response to injury may be part of neuroprotective responses. Indeed, after spinal cord transection of the male rat spinal cord, the administration of progesterone has been shown to prevent chromatolytic changes in the cytoplasm of the ventral horn motoneurons and to regulate the expression of genes involved in neuronal and glial functions and plasticity [1]. Protective effects of progesterone on motoneurons have also been demonstrated in the Wobbler mouse, a mutant characterized by spontaneous motoneuron degeneration and astrogliosis in the spinal cord and used as a model of amyotrophic lateral sclerosis (ALS) [234,235].

13. Changes in brain steroid levels in response to ischemic injury

Estradiol levels have been measured in plasma and in microdialysis samples taken from the parabrachial nucleus of male rat brains at different times after MCAO. Two interesting observations were made in this study: (1) estradiol levels in dialysates were greater than those measured in plasma; (2) the brain concentrations of estradiol rapidly increased after MCAO, already peaking after 10 min, but then decreasing to below pre-MCAO values at 90 min [236]. These results suggest that estradiol is released into the parabrachial nucleus in response to MCAO. Unfortunately, estradiol was measured with a chemiluminescent enzyme immunoassay kit.

Strong induction of aromatase expression in astrocytes of the rat hippocampus in response to global cerebral ischemia has been also reported. The induction of hippocampal aromatase was accompanied by an increase in estradiol levels, evaluated by ELISA and by estradiol immunostaining on frozen brain sections [51]. As for the immunohistochemical detection of allopregnanolone in brain section discussed above, the analysis of estradiol or other steroids by immunohistochemistry on tissue sections is questionable.

Errors inherent to immunological measures of steroids, and in particular their lack of specificity, are particularly problematic for clinical studies. They may indeed lead to inconsistent data with consequences for health-related decision making. Examples of recent studies that have suggested an association between ischemic stroke in postmenopausal women and endogenous estradiol or adrenal steroid levels measured by direct RIA or other types of immunoassays are eloquent [237,238].

In another experimental MCAO study, levels of progesterone were upregulated in both plasma and brain of male mice in response to sham surgery, reflecting adrenal stimulation by surgical stress. Plasma levels of corticosterone were indeed also significantly increased. Importantly, 6 h after MCAO, brain levels of progesterone and 5α-DHP were specifically upregulated, reaching momentarily a cumulative concentration of about 200 nM [239] (see Fig. 1 for the metabolism of progesterone). Thus, ischemic insult induces near "pregnancy levels" of progesterone and 5α-DHP in the male mouse brain. It is important to specify that steroids were analyzed by GC–MS with state-of-the-art sample workup in this study [63]. In contrast to progesterone and 5α-DHP, which are both ligands of the intracellular progesterone receptors (PR), brain levels of the $GABA_A$ receptor modulator allopregnanolone were not affected by MCAO [239].

That the marked and transient increase in brain progesterone and 5α-DHP indeed plays a key role in the resistance of brain tissue to ischemic damage was demonstrated indirectly by using PR knockout males. Ablation of the PR markedly increased the vulnerability of the male mouse brain to ischemic damage when compared to wild-type animals. In this experiment, no exogenous progesterone was administered, and it was concluded that the brains of the wild-type males were protected by the marked increase in their endogenous brain progesterone against ischemic injury [239]. These results make another strong case for an important role of CNS neurosteroids in endogenous neuroprotective mechanisms.

14. Conclusions

There exists an extensive literature dealing with changes in CNS steroid levels in response to environmental challenges, pathological conditions and lesions. These studies are covering a period of three decades, during which significant progress has been made in the development of new technologies for the analysis of steroids. Important milestones have been the introduction of mass spectrometry during the early sixties, the advent of RIA at the end of the same decade and the first analysis of brain steroids by GC–MS in 1995 [27,54,56]. One would logically expect improved precision of the reported measures of steroids in plasma and CNS tissue over time. This is partly true, as absolute brain steroid levels, which have been reported in recent studies using state-of-the art technologies, are often one order of magnitude less than those reported in the eighties or nineties. Moreover, it has been established that PREGS and DHEAS are not endogenous CNS steroids in rats and mice. On the other hand, journals have become surprisingly less demanding concerning the quality and validation of steroid assays, and steroid analysis by direct RIA or ELISA, methods that cannot provide accurate measures, are now frequently published.

It is not always easy to appreciate the accuracy of steroid levels reported in the literature, and a major aim of this review is to encourage a more critical reading. We also wish to draw attention to the absolute need for well-validated methods. For clinical trials, over- or underestimation of steroid levels may result in misleading conclusions. An important message is that even recent mass spectrometric methods have their limits, and they only become reliable tools if combined with careful sample preparation upstream of GC–MS(/MS) or LC–MS(/MS). It is thus very important to rapidly reduce the cholesterol content of samples, as its autoxidation can lead to the formation of steroids such as pregnenolone, DHEA and androstenediol, either in solution or in dry conditions [63]. Furthermore, mass spectrometric analysis is now a prerequisite for steroid analysis and profiling thanks to its analytical specificity and sensitivity.

In spite of these methodological considerations, there are now many studies consistent with the hypothesis that changes in CNS steroid levels may be part of endogenous adaptative, protective, rescue or even regenerative processes. Importantly, many of the studies quoted in this review use mass spectrometric methods, indicating that these demanding technologies are becoming more accessible to research laboratories, and they are also translating into clinical practice. The use of mass spectrometric methods is essential for the analysis of the very low levels of steroids in plasma and tissues of children and postmenopausal women (in the picomolar range) [42,45].

It is not always easy to determine whether changes in brain or spinal cord steroid levels involve the steroidogenic endocrine glands or the local synthesis of neurosteroids. Changes in brain

levels of progesterone and allopregnanolone in response to different types of acute stress may mainly reflect stimulation of the adrenal gland, as they can be prevented by adrenalectomy. This does of course not rule out a contribution of neurosteroids. On the other hand, increases in brain steroids in response to degenerative processes or injury may be part of protective, rescue or even regenerative processes as in the case of myelin repair. Most of these studies are correlative or only provide indirect evidence for the physiological functions of endogenous brain steroids. The strongest evidence for protective functions of a neurosteroid comes from experiments based on the inactivation of the aromatase by pharmacological or genetic tools [5]. Targeted and conditional inactivation of biosynthetic pathways will offer deeper insights into the pathophysiological functions of neurosteroids.

Interestingly, most of the studies addressing potential adaptative functions of brain steroids have so far focused on a very limited number of neuroactive steroids, in particular allopregnanolone, progesterone and estradiol. Steroid profiling in the CNS will certainly open new avenues, and it can be expected to reveal complex adaptations of steroid metabolomes to environmental challenges, disorders and injuries. However, steroid profiling by mass spectrometric methods still presents some limits. Indeed, because of the existence of numerous isomeric compounds, the analysis of steroids requires highly targeted methods with chromatographic separation and multiple reaction monitoring (MRM). Thus, in contrast to global metabolomics, a major limitation of the focused profiling of steroids by MS remains its hypothesis-driven approach [240]. However, MS and MS/MS spectra also provide structural information and can allow the identification of new compounds with the aid of reference libraries. The development of more global metabolomic approaches for the analysis of steroids is indeed emerging [241–243].

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