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Review

Progesterone and allopregnanolone in the central nervous system: Response to injury and implication for neuroprotection

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ABSTRACT

Progesterone is a well-known steroid hormone, synthesized by ovaries and placenta in females, and by adrenal glands in both males and females. Several tissues are targets of progesterone and the nervous system is a major one. Progesterone is also locally synthesized by the nervous system and qualifies, therefore, as a neurosteroid. In addition, the nervous system has the capacity to bio-convert progesterone into its active metabolite allopregnanolone. The enzymes required for progesterone and allopregnanolone synthesis are widely distributed in brain and spinal cord. Increased local biosynthesis of pregnenolone, progesterone and 5α-dihydroprogesterone may be a part of an endogenous neuroprotective mechanism in response to nervous system injuries. Progesterone and allopregnanolone neuroprotective effects have been widely recognized. Multiple receptors or associated proteins may contribute to the progesterone effects: classical nuclear receptors (PR), membrane progesterone receptor component 1 (PGRMC1), membrane progesterone receptors (mPR), and γ-aminobutyric acid type A ($GABA_A$) receptors after conversion to allopregnanolone. In this review, we will succinctly describe progesterone and allopregnanolone biosynthetic pathways and enzyme distribution in brain and spinal cord. Then, we will summarize our work on progesterone receptor distribution and cellular expression in brain and spinal cord; neurosteroid stimulation after nervous system injuries (spinal cord injury, traumatic brain injury, and stroke); and on progesterone and allopregnanolone neuroprotective effects in different experimental models including stroke and spinal cord injury. We will discuss in detail the neuroprotective effects of progesterone on the nervous system via PR, and of allopregnanolone via its modulation of $GABA_A$ receptors.

Contents

Abbreviations: 5α-DHPROG, 5α-dihydroprogesterone; CNS, central nervous system; $GABA_A$ receptors, γ-aminobutyric acid type A receptors; GC/MS, gas chromatography/mass spectrometry; 3α-HSOR, 3α-hydroxysteroid oxidoreductase; 3β-HSD, 3β-hydroxysteroid dehydrogenase; MCAO, middle cerebral artery occlusion; PGRMC1, membrane progesterone receptor component 1; mPR, membrane progesterone receptors; PR, progesterone receptors; PR-A, progesterone receptor isoform A; PR-B, progesterone receptor isoform B; PRE, progesterone response element; SRC1,2,3, steroid receptor coactivator-1,2,3; SCI, spinal cord injury; TBI, traumatic brain injury.

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1. Introduction

Progesterone is a hormone and a neurosteroid. As a hormone, it is synthesized by the ovaries, placenta and adrenal glands. As a neurosteroid, it is synthesized locally within the nervous system by both neurons and glial cells. In addition, progesterone can be metabolized into allopregnanolone (also named 3α,5α-tetrahydroprogesterone). The concentrations of these neurosteroids change under different physiological and pathological states [1–8]. Progesterone and allopregnanolone exert several effects in brain and spinal cord including neuroprotection. Their neuroprotective efficiencies have been demonstrated in different experimental models including traumatic brain injury (TBI), spinal cord injury, ischemic stroke, excitotoxic damage of hippocampal neurons and neurodegenerative diseases [9–26].

However, the mechanisms, targets, and effectors of the neuroprotective effects of progesterone and allopregnanolone are understudied. Clarifying their signaling mechanisms is a prerequisite for their efficient therapeutic use.

2. Progesterone and allopregnanolone in brain and spinal cord: sources, biosynthetic pathways and enzyme distribution

The synthesis of progesterone in the nervous system has been demonstrated in several species, and the enzymes required for progesterone and allopregnanolone synthesis are widely distributed throughout the brain and spinal cord [11,27–42]. Progesterone synthesis involves the conversion of cholesterol to pregnenolone by cytochrome P450scc enzyme (located in the mitochondria), then the conversion of pregnenolone to progesterone by the 3β-hydroxysteroid dehydrogenase (3β-HSD) enzyme (located in the mitochondria and the endoplasmic reticulum) [43,44]. The translocation of cholesterol inside the mitochondria is a rate -limiting step in the synthesis of steroids. It involves the "transduceosome", composed of multiple proteins including the translocase 18 kDa (TSPO, previously known as the peripheral benzodiazepine receptor), and the protein StAR [45].

In the brain, the expression of P450scc is well documented [36,46]. 3β-HSD mRNA showed a wide distribution throughout the rat brain. It is principally expressed in neurons of the olfactory bulb, striatum, cortex, thalamus, hypothalamus, septum, habenula, hippocampus and cerebellum [29]. This suggests a broad role of progesterone in regulating neural functions. *In vitro* studies using highly purified cell types have shown that both neurons and glial cells express P450scc and 3β-HSD and can convert pregnenolone to progesterone. Purkinje cells (a typical cerebellar neuron) have been shown to express P450scc and 3β-HSD during postnatal development and in the adulthood and were demonstrated to be a source of progesterone [47,48]. The brain has also the capacity to metabolize progesterone; the major metabolic pathway in the nervous system is 5 α-reduction [49–54]. Progesterone is first converted by the enzyme 5α-reductase into 5α-dihydroprogesterone, which is converted into allopregnanolone by the 3α-hydroxysteroid oxidoreductase (3α-HSOR) enzyme [55,56]. In mouse and rat brain, these enzymes have been shown to be co-expressed in neurons. *In situ* hybridization combined with immunofluorescence analysis allowed the identification of these neurons as mainly glutamatergic neurons in the cortex, hippocampus and olfactory bulb and as GABAergic output neurons in the striatum, thalamus and cerebellum [52].

In the spinal cord, significant levels of pregnenolone and progesterone remained detectable after the removal of gonads and adrenal glands suggesting their local synthesis [53,57]. Several groups have shown that spinal cord possesses the enzymatic set to synthesize pregnenolone, progesterone and allopregnanolone [51,53,57–60]. P450scc enzyme is expressed and is bioactive in the dorsal horn, nociceptive supraspinal nuclei and somatosensory cortex [59,60]. 3β-HSD mRNA is expressed in motoneurons in the ventral horn and small neurons of the dorsal horn [57]. 5α-reductase type 1 is expressed only by glial cells while 5α-reductase type 2 and 3α-HSOR are expressed by oligodendrocytes, neurons and astrocytes [51].

3. Progesterone receptors: PR, PGRMC1 and mPR in brain and spinal cord

Progesterone uses multiple signaling pathways: the classical one is the regulation of gene expression after binding to intracellular progesterone receptors (PR) which belong to the nuclear receptor super-family of transcription factors. Progesterone may also bind to specific membrane sites (mPR/PGRMC1 complex) and activate intracellular signaling pathways. Finally, progesterone may modulate $GABA_A$ receptor activity after conversion to allopregnanolone [11,61–65]. Both brain and spinal cord express all these receptors and have the capacity to metabolise progesterone into allopregnanolone. This suggests that, one or the other mechanism may be triggered in these tissues according to the physiological or pathological conditions, depending on the available concentration and the expression of the different receptors.

3.1. Intracellular progesterone receptors (PR)

The transcriptional ("genomic" or "classical") effects of progesterone are mediated by at least two intracellular receptor isoforms (PR-A and PR-B). They are transcribed from two distinct promoter regions of a single gene and differ by an additional 164-amino-acid segment in the N-terminal region of PR-B [66–68]. Four functional domains can be distinguished in the structure of PR: the N-terminal domain, the DNA binding domain (DBD), the hinge region and the ligand binding domain (LBD) at the C-terminus. PR contain also a transcription activation function (AF) domain and inhibition function (IF) domains [69]. According to the classical view, gene transcription is activated by PR dimers bound to palindromic response elements (PRE) in the promoter region of the target genes. The detailed PR signaling was recently reviewed in [11].

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PR expression is regulated by estrogens and progesterone. Estrogens induce PR but not in all brain regions. Indeed, estrogen stimulates PR expression in the hypothalamus [70–72] but not in cerebral cortex, septum, caudate putamen, supraoptic nucleus, dentate gyrus, the CA3 region of the hippocampus, midbrain, and cerebellum [70–72]. PR expression is only moderately induced by estrogen in other hypothalamic and limbic regions such us bed nucleus of the stria terminalis, amygdaloid nucleus and the CA1 region of the hippocampus. Progesterone down-regulates PR which are inducible by estrogen but not those which do not respond to estrogen. Indeed, in both the frontal cortex and cerebellum progesterone does not alter PR expression [73–75].

The expression and regulation of PR-A and PR-B isoforms in the brain are different [74–77]. In male rat brain, PR-A expression is higher than that of PR-B in the hypothalamus, while PR-B expression is predominant in the cerebellum. PR isoforms are expressed at similar levels in the frontal cortex and the olfactory bulb [77]. Oestradiol induces PR-A in the cerebellum of gonadectomised male rat. In contrast, in ovariectomised female rats, oestradiol induces both PR-A and PR-B isoforms in the hypothalamus, PR-B isoform in the preoptic area, and PR-A isoform in the hippocampus and the olfactory bulb. Oestradiol does not modify the expression of PR-A and PR-B isoforms in the cerebellum and the frontal cortex. Progesterone down-regulates both isoforms in the hypothalamus, PR-B isoform in the pre-optic area, and PR-A isoform in the hippocampus. Progesterone does not modify PR isoforms expression in olfactory bulb, cerebellum and frontal cortex [74,75].

The steroid receptor coactivators SRC-1, SRC-2, and SRC-3 play an important role in steroid receptor-dependent gene expression and show a wide distribution in brain including hypothalamus, cortex, hippocampus, cerebellum, and brain stem [78–83]. They have been shown to regulate neural plasticity [84,85] and to be involved in some neurodegenerative diseases and brain injuries [86–88].

In spinal cord, PR are expressed in motoneurons, glial cells and ependymal cells of the central canal and their expression is not affected by estrogen treatment [89]. After spinal cord transection, the expression of PR decreased and progesterone administration did not affect PR levels [90]. Several nuclear steroid receptor activators have been shown to be expressed in motoneurons [91]. SRC-1 was predominant in neurons of the superficial laminae of the dorsal horn and within motorneurons of lamina IX [92].

3.2. PGRMC1

The first potential membrane receptor of progesterone was cloned from porcine liver microsomes [93]. Its rat analog is the protein 25-Dx [94] renamed progesterone receptor membrane component 1 (PGRMC1). Krebs and all have shown that one interesting mRNA within the hypothalamus that is repressed by progesterone after priming with estrogens, encodes the protein PGRMC1 [95]. PGRMC1 is expressed in the brain and in the spinal cord [90,95–97] and its expression is up-regulated after injury [90,96]. In brain, PGRMC1 is particularly abundant in the hypothalamus, circumventricular organs, ependymal cells of the ventricular walls, and the meninges. Interestingly, it is co-expressed with vasopressin in neurons of the paraventricular, supraoptic and retrochiasmatic nuclei. In response to TBI, PGRMC1 expression is up-regulated in neurons and induced in astrocytes [96]. The expression of PGRMC1 in structures involved in cerebrospinal fluid production and osmoregulation, and its up-regulation after brain damage, point to a potentially important role of this progesterone receptor membrane component in the maintenance of water homeostasis after TBI. In spinal cord, PGRMC1 is expressed in neurons of the dorsal horn and ependymal cells lining the central canal. A role of PGRMC1 in mediating the protective effects of progesterone in the spinal cord is supported by the observation that its mRNA and protein are up-regulated by progesterone in dorsal horn of the injured spinal cord [90].

A variety of functions have been proposed for PGRMC1, including acting as a component of a membrane progestin receptor and as an adaptor protein. PGRMC1 does not appear to function as a traditional receptor because it requires a binding partner known as serpine mRNA binding protein 1 [98]. Using photoaffinity probe Xu et al. identified the PGRMC1 protein complex as the putative sigma-2 receptor-binding site. Immunocytochemistry revealed that both PGRMC1 and SW120, a fluorescent sigma-2 receptor ligand, colocalized with molecular markers of the endoplasmic reticulum and mitochondria [99]. Recent data suggest that PGRMC1 can act as an adaptor protein, transporting mPRα to the cell surface, and that PGRMC1 and mPRα are components of a membrane progesterone receptor protein complex [100]. Indeed, stable overexpression of human PGRMC1 in PR-negative breast cancer cell lines caused increased expression of PGRMC1 and mPRα on cell membranes that is associated with increased specific [^{3}H]progesterone binding [100].

3.3. Membrane progesterone receptors (mPR)

Progesterone may also act after binding to mPRα, mPRβ and mPRγ membrane progesterone receptors, distinct from the classical PR, cloned by Thomas and co-workers [101,102]. A recent work from the same group characterized two other mPR types: human mPRδ and mPRε [103]. mPRδ and mPRε activate a stimulatory G protein (Gs), unlike the other mPRs, which activate an inhibitory G protein (Gi). All five mPR mRNAs were detected in the human brain and spinal cord with some differences in their expression levels and distribution patterns. mPRδ showed the highest level of expression among all the other isoforms, followed by mPRβ. Variables levels of expression were observed for mPRα and mPRγ. mPRα was detected in many regions and the highest levels were observed in the temporal lobe, pituitary gland, medulla, and spinal cord. mPRβ is highly expressed in spinal cord and many brain regions including the corpus callosum, hippocampus, hypothalamus, substantia nigra, cerebellum. mPRγ showed a lower expression than that of other mPRs in many brain regions, and highest relative levels were observed in spinal cord, choroid plexus, and pons. mPRδ showed a greatest expression in the forebrain, hypothalamus, amygdala, corpus callosum, and spinal cord, whereas mPRε was abundant in the pituitary gland and hypothalamus [103].

We have recently reported the wide distribution of mPRα in the mouse and rat brain [104]. Indeed, mPRα is expressed in the olfactory bulb, striatum, cortex, thalamus, hypothalamus, septum, hippocampus, and cerebellum. Double immunofluorescence and confocal microscopy analysis showed that mPRα is expressed by neurons, but not by oligodendrocytes and astrocytes (Fig. 1). In the rat brain, the distribution and cell type localisation of mPRα was similar to that observed in mouse brain. However, after focal TBI, mPRα expression was strongly induced in oligodendrocytes, astrocytes and reactive microglia (Fig.2). The wide neuroanatomical distribution of mPRα suggests that this receptor may play a role beyond neuroendocrine and reproductive functions. However, in the absence of injury its role might be restricted to neurons. The induction of mPRα after TBI in glial cells points to a potential role in mediating the modulatory effects of progesterone on inflammation, ion and water homeostasis and myelin repair in the injured brain. mPRβ has been detected in the mouse and rat brain [105]. Expression of mPRβ mRNA has been more specifically localized to the medio-basal hypothalamus [106,107]. However, another study

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showed that its expression was higher in thalamic nuclei and cortex rather than in hypothalamus [108]. A more recent study, using immunohistochemistry showed a wide distribution of mPRβ protein in the female rat brain. mPRβ was highly expressed in hypothalamus, forebrain, midbrain, cortex, and thalamus and its expression was increased by estradiol within the medial septum [109]. The expression of mPRγ in mice brain is low, it was detected by RT-PCR but could not be mapped by in situ-hybridization (Meffre and Guennoun, unpublished results).

mPRα is widely distributed throughout the mouse spinal cord. However, in contrast to the brain, the receptor is not only expressed in most neurons, but also in astrocytes, oligodendrocytes and $NG2^+$ oligodendrocyte progenitor cells, even in the absence of a lesion. mPRβ showed a more restricted distribution, and was mainly present in ventral horn motoneurons and in neurites, but not in glial cells. These observations suggest that the two mPR isoforms may mediate distinct and specific functions of progesterone in the spinal cord. Although mPRγ mRNA was detected in spinal cord tissue by RT-PCR, *in situ* hybridization analysis did not allow us to verify and to map its presence. A significant observation in both brain and spinal cord was the very stable expression of mPRα. Indeed, the expression of mPRα was similar in both sexes and was not influenced by the presence or absence of the classical PR. Treatment of males by estradiol or progesterone did not modify the level of expression of mPRα [104,110].

4. Mechanisms of action of allopregnanolone

In contrast to its precursors (progesterone and 5α-dihydroprogesterone), allopregnanolone does not bind to the classical intracellular progesterone receptors PR. Allopregnanolone is a potent endogenous positive modulator of the $GABA_A$ receptor, the principal mediator of the fast inhibitory transmission within the central nervous system. Two sites on the $GABA_A$ receptor complex to which allopregnanolone binds, were identified [111]. Allopregnanolone prolongs the opening time of chloride ion channels in $GABA_A$ receptors and enhances the inhibitory transmission. Synaptic $GABA_A$ receptors mediate rapid phasic inhibition of postsynaptic currents, while the activation of extrasynaptic $GABA_A$ receptors, results on tonic inhibition [112–114]. Phasic inhibition occurs when high levels of GABA, rapidly activate post-synaptic $GABA_A$ receptors resulting in a transient inhibitory response. Tonic inhibition is observed when continuous low levels of GABA activate extra-synaptic $GABA_A$ receptors and generate persistent inhibition of neuronal excitability. Allopregnanolone potentiates both phasic and tonic inhibition by modulating synaptic and extrasynaptic $GABA_A$ receptors [112–114]. The subunit composition, pharmacological, and functional properties of extrasynaptic and synaptic $GABA_A$ receptors are different [112,115]. Indeed, the γ2 subunit contributes to the postsynaptic localization of $GABA_A$ receptors, whereas δ subunit containing receptors are predominantly extrasynaptic [116,117]. The extra-synaptic receptors, containing

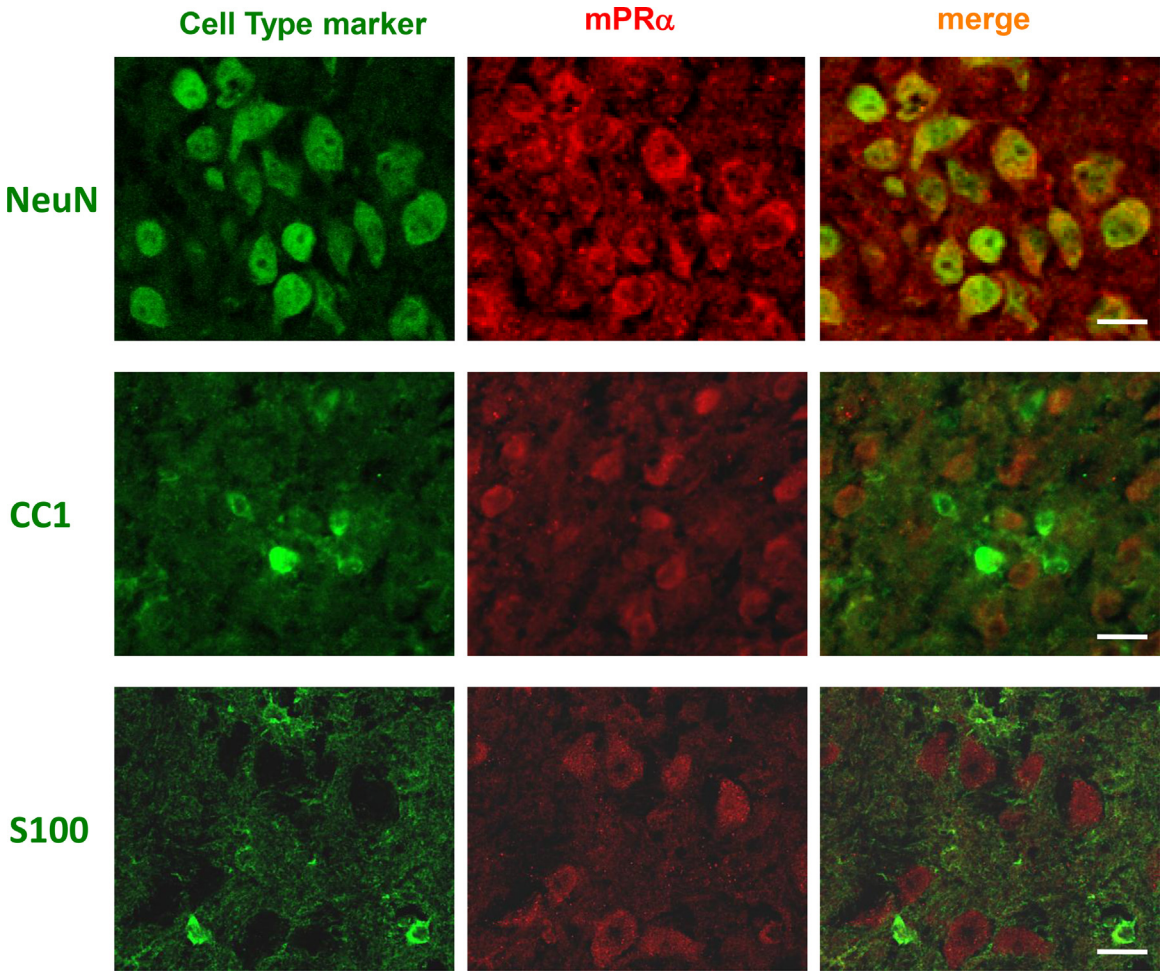

Fig. 1. Phenotypical characterisation of cells expressing mPRα in brain; analysis by double immunofluorescence and confocal microscopy. mPRα is expressed by neurons (mPRα/NeuN colocalization) and is not expressed by oligodendrocytes (no colocalisation of mPRα/CC1) or astrocytes (no colocalisation of mPRα/S100). Scale bar = 30 μm. Data from Meffre et al. [104].

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the δ subunit, are insensitive to a variety of benzodiazepines, but are sensitive to allopregnanolone [113,118]. Accordingly, δ subunit $GABA_A$ receptors knockout mice show reduced sensitivity to allopregnanolone [118,119]. In addition to the differential sensitivity linked to sub-unit composition, there are differences linked to the regional brain localisation. Thus, extrasynaptic $GABA_A$ receptors in thalamocortical neurons, were relatively insensitive to neurosteroids [115]. Variations in brain levels of allopregnanolone may produce important changes in neuronal excitability. Within the dentate gyrus of the hippocampus, allopregnanolone is actively metabolised and GABAergic synapses have low sensitivity to this neurosteroid. In contrast, within the CA1 region of the hippocampus, allopregnanolone metabolism is less active and neurons respond to low concentrations of allopregnanolone. These findings suggest a crucial role for local steroid metabolism in regulating $GABA_A$ receptor-mediated inhibition in a regionally dependent

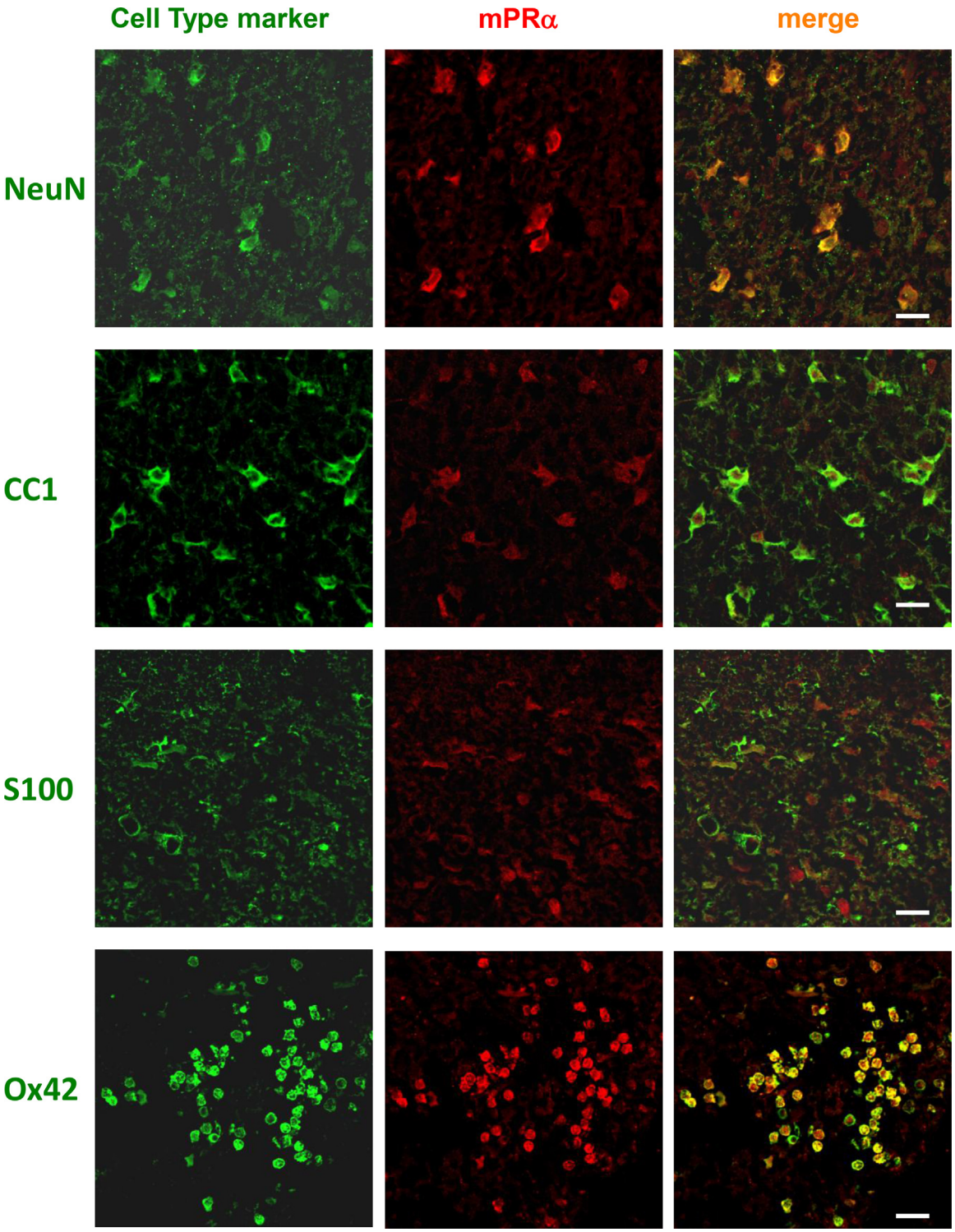

Fig. 2. Effects of traumatic brain injury on mPRα expression: analysis by double immunofluorescence and confocal microscopy. After TBI, mPRα was still expressed in neurons (mPRα/NeuN colocalization) and is induced in oligodendrocytes (mPRα/CC1 colocalization), astrocytes (mPRα/S100 colocalization) and reactive microglia (mPRα/Ox42 colocalization). Scale bar = 30 μm. data from Meffre et al. [104].

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manner [120]. In spinal cord, a recent study has demonstrated that the spatially restricted synthesis of allopregnanolone determines differences in synaptic $GABA_A$ receptor activity, which is observed between different layers of neurons in dorsal horns of the rat spinal cord during postnatal development [121].

Recent patch-clamp studies conducted in our laboratory cast a new light on the significance of allopregnanolone in the central nervous system. They show that the modulation of $GABA_A$ receptors by allopregnanolone also influences the synaptic release and activity of glycine, a major inhibitory neurotransmitter. These findings point to a new mechanism by which this neurosteroid may protect neuronal networks against excitotoxic damage [122,123].

In addition to the potentiation of $GABA_A$ signaling, other targets of allopregnanolone have been identified. The pregnane X receptor (PXR) has been proposed to mediate part of the neuroprotective effects of allopregnanolone in a mouse model of Niemann–Pick type C disease [124]. PXR acts as a ligand-activated transcription factor, and regulates the expression of genes involved in xenobiotic detoxification and apoptosis [125]. Allopregnanolone also binds to membrane progesterone receptors (mPR) [102,126]. Allopregnanolone has been shown to act as a potent mPRα- agonist on the immortalized hypothalamic neuronal cell line GT1-7. At low nanomolar concentrations, allopregnanolone mimicked the actions of progesterone: decreased cAMP accumulation and anti-apoptotic effects [127]. Recently, allopregnanolone has been shown to bind to mPRδ and other mPRs and to act as agonist, activating second messengers and decreasing starvation-induced cell death and apoptosis in mPRδ -transfected cells and in hippocampal neuronal cells at low nanomolar concentrations. These results suggest that mPRδ is a potential intermediaries of nonclassical antiapoptotic actions of neurosteroids in the central nervous system [103]. mPRs could mediate neuroprotective actions of allopregnanolone, in addition of those of progesterone, throughout the central nervous system, as they are widely and abundantly distributed in rat, mouse and humans brain and spinal cord [103,104,110].

5. Progesterone and allopregnanolone levels in brain and spinal cord: response to injury

The concentrations of progesterone and allopregnanolone in nervous tissues are not static; they change dynamically under different physiological and pathological states. In particular, their levels change during the reproductive cycle and during pregnancy, and in response to challenges as stress, neurological diseases, or injury [1–8,128]. We will focus here on our recent results concerning the effect of spinal cord injury, traumatic brain injury and cerebral ischemia on pregnenolone, progesterone, 5α-dihydroprogesterone and allopregnanolone levels. It is important to note that steroid analysis was performed by gas chromatography/mass spectrometry (GC/MS) with an improved tissue extraction method [129,130]. Results are summarised in Table 1.

5.1. Effect of spinal cord injury on endogenous spinal cord steroid levels

The levels of pregnenolone, progesterone, 5α-dihydroprogesterone, and allopregnanolone increased in rat spinal cord 75 h after transection without significant increases in the plasma. After combined adrenalectomy and gonadectomy, significant levels of pregnenolone and progesterone remained in the spinal cord, suggesting their local biosynthesis. In the spinal cord of adrenalectomized and gonadectomized rats, there was an increase of pregnenolone 24 h after spinal cord injury, followed at 75 h by an increase in its direct metabolite, progesterone. These observations are consistent with a sequential increase of pregnenolone biosynthesis and its conversion to progesterone, within the spinal cord in response to injury. However, no significant change in P450scc and 3β-HSD mRNA levels was observed after spinal cord injury suggesting a stimulation of the activities rather than a stimulation of the expression of these steroidogenic enzymes [53].

5.2. Effect of traumatic brain injury (TBI) on endogenous brain steroid levels

In TBI, the endogenous circulating hormones at the time of injury are important for neuroprotection. In particular, pseudopregnant female rats recover better than males from TBI [131,132]. We have investigated the effect of pseudopregnancy and TBI on plasma and brain steroid levels. We found different steroid profiles in male and pseudopregnant female rats and specific profile changes after TBI. In sham-operated pseudopregnant females, much higher levels of progesterone, 5α-dihydroprogesterone, allopregnanolone and epiallopregnanolone were measured in both brain and plasma, compared with sham-operated males. 6 h after TBI, the levels of pregnenolone, progesterone, and 5α-dihydroprogesterone increased, and those of testosterone decreased in male brain, whereas levels of 5α-dihydroprogesterone and epiallopregnanolone increased in the brain of pseudopregnant female rats. Plasma levels of 5α-dihydroprogesterone did not change after TBI, suggesting a local activation of the 5α- reduction pathway of progesterone in both male and pseudopregnant female brain [54]. The significant increase in neurosteroid levels in the male brain after TBI is consistent with their role in neuroprotection. In pseudopregnant females, high levels of circulating progestogens may provide higher protection against TBI comparatively to males.

Table 1
Steroid levels in brain and spinal cord are up-regulated in response to CNS injuries as analysed by GC/MS.

Steroids Experimental mode	Pregnenolone	Progesterone	5α-DHPROG	Allopregnanolone	References
SCI (male rats) 75th post-injury	↑	↑	↑	↑	[53]
SCI (ADX/GDX male rats)24th post-injury	↑	–	–	–	[53]
75th post-injury	–	↑	–	–	
TBI (male rats) 6th post-injury	↑	↑	↑	–	[54]
MCAO (male mice) 6th post-injury	↑	↑	↑	–	[22]

Abbreviations: ADX/GDX: adrenalectomy/gonadectomy; CNS: central nervous system; 5α-DHPROG: 5α-dihydroprogesterone; GC/MS: gas chromatography/mass spectrometry; MCAO: middle cerebral artery occlusion, an experimental stroke model; SCI: spinal cord injury; TBI: traumatic brain injury.

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5.3. Effects of cerebral ischemia on endogenous brain steroid levels

Levels of progesterone were strongly increased, in the brains of male mice as early as 6 h after middle cerebral artery occlusion (MCAO), reaching levels observed in females during pregnancy. The increase in brain progesterone was observed both in the lesioned ipsilateral and in the contralateral brain sides. We also observed a 2- to 3-fold increase in brain levels of the progesterone metabolite 5α-dihydroprogesterone, which also increases the transcriptional activity of PR [133]. In contrast, stroke did not significantly affect brain levels of allopregnanolone, which were about 10 times lower than those of progesterone and 5α-dihydroprogesterone. A part of the progesterone present in brain tissue may be of adrenal origin, because plasma levels of the hormone were increased in response to surgical stress. However, ischemic injury specifically stimulated an important increase in the brain levels of progesterone and 5α-dihydroprogesterone, which were not observed in plasma. Moreover, the ratios of brain to plasma levels of progesterone and 5α-dihydroprogesterone were significantly increased after MCAO, but not in response to sham surgery. In response to ischemic injury, brain levels of progesterone plus 5α-dihydroprogesterone reached about 200 nM levels in the male brain, whereas levels of allopregnanolone remained in the low nanomolar range [22]. Up-regulation of the brain's endogenous PR ligands (progesterone and 5α-dihydroprogesterone) may thus confer resistance to ischemic damage. The transient increase in endogenous brain progesterone in response to ischemic injury may contribute to the extended window for the neuroprotective efficacy of progesterone treatment, up to 6 h [134,135].

Our results (Table 1), discussed above, show that levels of pregnenolone, progesterone and 5α-dihydroprogesterone are strongly up-regulated in spinal cord in response to spinal cord injury [53], in brain after TBI [54], and after ischemic brain injury [22]. This increase of neurosteroids in response to injury may be part of the responses of neural cells to damage and degenerative processes, and may be an endogenous physiological process. This is an important concept because: (1) it suggests that neuroprotective effect of progesterone treatment may simply be a means of boosting this natural endogenous protective process; (2) the increase in endogenous central nervous system progesterone levels may be behind the relatively large window of therapeutic opportunity after injury; (3) it may be possible to extend the therapeutic window by stimulating neurosteroid synthesis.

6. Neuroprotective effects of progesterone and allopregnanolone

Progesterone exerts marked neuroprotective effects in the injured spinal cord. After spinal cord transection, progesterone prevents chromatolysis, preserves motoneurons ultrastructure and up-regulates the expression of BDNF and choline acetyltransferase [13] [136] Progesterone affects also glia: it decreases the proliferation and activation of astrocyte and microglia, and increases the proliferation and differentiation of oligodendrocyte progenitor cells [137,138]. In an experimental model of spinal cord contusion, progesterone treatment reduces the volume and extension of the lesion, increases the number of oligodendrocytes, up-regulates myelin basic protein, increases the number of axonal profiles and improves locomotor outcome [139]. Neuroprotective effects of progesterone were also shown in a mouse model of spontaneous spinal motoneuron degeneration, the Wobbler mice. Treatment with progesterone increases muscle strength, biceps weight and survival. At the cellular level, progesterone treatment reduces motoneuron vacuolation, restores BDNF and choline acetyltransferase expressions, decreases the activity of NOS and enhances complex I respiratory activity and MnSOD2, and reduces astrogliosis [140,141,13,142]. Stein et al. conducted most studies showing the neuroprotective effects of progesterone after TBI [12,132,143]. A wide range of actions underlying the neuroprotective effects of progesterone has been demonstrated. Progesterone reduces brain edema [144,145], inflammation [146,147] and oxidant activity [14]. Progesterone preserves mitochondrial functions [148,149] and regulates hemostatic proteins [150], and promotes the survival of newborn neurons [151]. A systematic meta-analysis has confirmed the efficiency of progesterone in TBI [134]. Two phase II clinical trials have shown that progesterone treatment induces a significant improvement in patients with TBI [152,153].The signaling mechanisms involved in the neuroprotective effects of progesterone in TBI are understudied. The prevailing view is that they may be mainly mediated by allopregnanolone. This hypothesis is based on studies showing that allopregnanolone treatment can mimick the effects of progesterone [15,147]. However, our results on PGRMC1 and mPRα expressions suggest a potentially important role of PGRMC1 in the maintenance of water homeostasis and a potential role of mPR α in mediating the modulatory effects of progesterone on inflammation, ion and water homeostasis and myelin repair after TBI [96,104]. Several studies have demonstrated the neuroprotective effects of progesterone after cerebral ischemia. Progesterone treatment reduces lesion volume, inflammatory responses, decreases blood brain barrier permeability and improves functional outcome [19,22,134,149,154–165]. Progesterone treatment provides long-term protection for both males and females [134,166,167]. Two-meta analysis provided supporting evidence for the efficiency of progesterone in reducing lesion volume after ischemia [134,168].

Allopregnanolone has been shown to be neuroprotective in different experimental models such as TBI, ischemia, neurodegenerative diseases and spinal cord injury [15,16,23,149]. Several studies have shown the neuroprotective effects of allopregnanolone in experimental models of TBI [15,147,149]. A phase II clinical trial to test the safety and effectiveness of allopregnanolone in improving neurobehavioral outcome and reducing mortality in adults with moderate and severe traumatic brain injury is ongoing (http://clinicaltrials.gov/ct2/show/NCT01673828?term=traumatic+brain+injury+and+allopregnanolone&rank=1 (2012). The administration of allopregnanolone was more potent than progesterone (at the same dose) in reducing volume infarct after cerebral ischemia [16]. Furthermore, allopregnanolone, inhibits the activity of the mitochondrial permeability transition pore, a key player in apoptosis, while progesterone was without effect [149]. Like progesterone, allopregnanolone treatment reduces dysfunctions of the blood- brain -barrier and neuroinflammation [165]. The neuroprotective effects of allopregnanolone have also been demonstrated in a mouse model of Niemann–Pick type C (NP-C) disease. Indeed, a single administration of allopregnanolone significantly delays Purkinje neuron death, the loss of locomotion and coordination, and the death of the animals [18]. Allopregnanolone provides also neuroprotection in a transgenic mouse model of Alzheimer's disease (3xTgAD mice) [9]. In the 3xTgAD mice, allopregnanolone treatment stimulates neurogenesis and survival of newly generated cells within the hippocampus, and restores learning and memory functions [169,170]. At the cellular level, allopregnanolone increases the expression of the 2′,3′-cyclic-nucleotide 3′-phosphodiesterase (CNPase), a specific marker of oligodendrocytes and reduces amyloid-β peptide generation and neuroinflammation [171]. Allopregnanolone induces the proliferation of rat and human neural stem cells and embryonic rat hippocampal neurons [170,172]. Allopregnanolone treatment has been shown to have neuroprotective benefits in other experimental models of neurodegenerative diseases including multiple sclerosis and Parkinson [173,174].

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Table 2
Neuroprotective effects of progesterone and of allopregnanolone and role of intracellular receptors (PR) and $GABA_A$ receptors.

Neuroprotection In	Progesterone signaling via PR: experimental evidence	Allopregnanolone signaling via $GABA_A$ receptors independently of PR: experimental evidence	References
MCAO model	- Increased sensitivity to brain ischemic damage in $PR^{-/-}$ mice. - Progesterone treatment fails to provide neuroprotection after MCAO in $PR^{-/-}$ mice. - Nestorone provides neuroprotection.	- Allopregnanolone treatment protects the brain of $PR^{-/-}$ mice against ischemic damage.	[22]
Spinal cord injury	- Progesterone does not protect neurons in organotypic spinal cord slice cultures of $PR^{-/-}$ mice.	- Allopregnanolone protects neurons in spinal cord slice cultures of $PR^{-/-}$ mice. - Gabazine inhibits the effect of allopregnanolone.	[23]
Injury by excitotoxicity or toxins		- Progesterone treatment increases the synthesis of allopregnanolone in the hippocampus and protects neurons from death by kainic acid or by tributyltin. - Finasteride or bicuculline, abolished the neuroprotective effect of progesterone	[24–26]

Abbreviations: MCAO: middle cerebral artery occlusion, an experimental stroke model; PR: classical intracellular progesterone receptors; $PR^{-/-}$ mice: refers to total PR knockouts.Bicuculline is a potent $GABA_A$ receptor antagonist; finasteride is an inhibitor of 5α-reductase; Nestorone is a potent and selective PR agonist, Gabazine is a selective $GABA_A$ receptor antagonist.

The signaling mechanisms underlying the neuroprotective effects of progesterone and allopregnanolone are under-studied. However, in some experimental models a key role of PR and $GABA_A$ receptors have been demonstrated (Table 2).

6.1. Progesterone receptor-dependent neuroprotection after stroke and spinal cord injury

Our recent experimental results demonstrated for the first time a key role for the intracellular progesterone receptor (PR) in the protection of the brain against stroke-induced damage [22]. This was demonstrated in an experimental model of transient middle cerebral artery occlusion (MCAO) using wild-type $PR^{+/+}$, heterozygous $PR^{+/-}$, and homozygous knockout $PR^{-/-}$ knockout adult male mice [175]. In response to the occlusion, the infarct volume was much larger in heterozygous $PR^{+/-}$, and homozygous knockout $PR^{-/-}$ mice than in wild-type $PR^{+/+}$ mice indicating that PR expression is a limiting factor for the neuroprotective efficacy of endogenous progesterone. The greater resistance of wild-type $PR^{+/+}$ mice to ischemia comparatively to $PR^{+/-}$ and $PR^{-/-}$ mice was observed at 6 h and 24 h after MCAO, but no longer after 48 h. These results demonstrated a key neuroprotective role of endogenous progesterone via the intracellular receptors PR. However, this effect is time-limited. Reducing the extent of brain tissue damage and the impairment of motor functions for a longer period, up to 48 h, indeed required the administration of exogenous progesterone. Consistent with a key role of PR, progesterone treatment was efficient in wild-type $PR^{+/+}$ mice but was not protective in $PR^{-/-}$ knockout mice [22]. It can also be concluded that endogenous conversion of progesterone to allopregnanolone is not sufficient for efficient neuroprotection, otherwise the administration of progesterone would have reduced infarct volume and improved neurological outcomes also in $PR^{-/-}$ mice [22].

The demonstration that the intracellular progesterone receptors (PR) play a key role in neuroprotective mechanisms after stroke opened new perspectives for the therapeutic use of potent and PR-selective synthetic progestins such as Nestorone in stroke treatment. Nestorone has indeed been designed to target selectively the PR with no unwanted interaction with other receptors. It can be used at a therapeutic dose 100-times lower than that of natural progesterone given its superior activity on the PR, and clinical studies conducted in men and women have shown that it is very well tolerated [176,177]. Using the MCAO experimental model, we have shown that the very potent and selective progestin Nestorone protects the brain against ischemic damage and improves functional outcomes at a very low dose [22].

Progesterone treatment also provides neuroprotection in the spinal cord. After experimental spinal cord transection, we have demonstrated a decrease in brain-derived neurotrophic factor mRNA, Na,K-ATPase mRNA, microtubule-associated protein 2 and choline acetyltransferase and that progesterone treatment restored the expression of these genes in motoneurons [13]. To test whether PR play a key role in the neuroprotective effects of progesterone in the spinal cord, as they do in the brain after ischemic injury, we used organotypic spinal cord slice cultures prepared from 3 weeks-old mice. This type of cultures offers an integrated *in vitro* system, accessible for pharmacological manipulations. Injury was induced by dropping a weight on the slices, resulting in a decreased number of neurons, and in particular of motoneurons. This was reflected by an increased number of dying cells, becoming permeable to propidium iodide (PI), and greater release of lactic acid dehydrogenase by the damaged plasma membranes. Adding progesterone, to the culture medium at the time of injury rescued the spinal cord slices from the effects of damage. These effects were not due to the conversion of progesterone to 5α-dihydroprogesterone or allopregnanolone, as they were not prevented by finasteride, an inhibitor of the 5α-reductase enzymes. The neuroprotective effects of progesterone required the presence of PR, as they could not be observed in slices from PR knockout ($PR^{-/-}$) mice [23].

6.2. Progesterone metabolism to allopregnanolone may participate to its neuroprotective effect in experimental models of neurodegeneration

Although our work has demonstrated a key role of PR in mediating the neuroprotective effects of progesterone after cerebral ischemia and spinal cord injury [22,23], the importance of modulatory effects of allopregnanolone should not be underestimated. Allopregnanolone may indeed contribute to the neuroprotective efficacy of progesterone via the modulation of $GABA_A$ receptors, as has been documented in other experimental studies. Thus, in two models of induced neuronal death by kainic acid [24,25] and by tributyltin [26] progesterone metabolism into allopregnanolone have been shown to be important for neuroprotection. Indeed, in these models, levels of allopregnanolone increased after progesterone administration. Furthermore, the use of finasteride to block allopregnanolone biosynthesis or bicuculline, a potent $GABA_A$ receptor antagonist, abolished the neuroprotective effect of progesterone.

6.3. Neuroprotective effects of allopregnanolone are not dependent on PR and involve the modulation of $GABA_A$ receptors

Allopregnanolone has been shown to be neuroprotective in different animal models such as TBI, ischemia, neurodegenerative

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diseases and spinal cord injury [15,16,149]. Using a transient MCAO model and PRKO mice, we have shown that treatment with allopregnanolone markedly reduced total infarct volume, ischemic damage in cerebral cortex and subcortical structures, and the extension of lesions at different brain levels in both $PR^{+/+}$ and $PR^{-/-}$ mice. Allopregnanolone also reduced brain edema independently of the genotype. The brain-protective effects of allopregnanolone were accompanied by improved functional outcomes evaluated on the rotarod, not only in wild-type $PR^{+/+}$, but also in $PR^{-/-}$ mice. Thus, allopregnanolone treatment can protect the brain against ischemic damage by signaling mechanisms which are not involving PR [22].

In spinal cord, using an *in vitro* model of neurotrauma we have shown recently for the first time the neuroprotective effects of the 5α-reduced derivatives of progesterone (5α-dihydroprogesterone and allopregnanolone). The effects of progesterone, 5α-dihydroprogesterone and allopregnanolone in preventing neuron loss were similar. The neuroprotective effects of allopregnanolone were not due to its bioconversion back to 5α-dihydroprogesterone, which can activate gene transcription via PR, because they were still observed in slices from knockout $PR^{-/-}$ mice. The effects of allopregnanolone involved $GABA_A$ receptors, as they were inhibited by the selective $GABA_A$ receptor antagonist Gabazine, in both $PR^{+/+}$ and $PR^{-/-}$ mice. This suggest that allopregnanolone, by modulating $GABA_A$ receptors, may have an anti-excitotoxic effect, which protects neurons from death after spinal cord injury.

7. Summary and therapeutic options

A key issue in neuroprotective and neuroregenerative strategies is the identification of the right drug targets and the understanding of underlying signaling mechanisms. Wrong choices and the lack of knowledge concerning mechanisms indeed lead to turns in the wrong direction for therapeutic efforts. Many drugs have failed in clinical trial because they act only at one or a few receptor sites. Progesterone and allopregnanolone are pleiotropic agents using different mechanisms of actions (Fig. 3); they are more likely to have a beneficial outcome than an agent that acts only at a single target. The natural neurosteroids, progesterone and allopregnanolone, or their synthetic analogs should be considered for therapeutical development as useful neuroprotectants.

Several studies have demonstrated the safety of progesterone for clinical use [178] while data showing safety, tolerability, and potential efficacy of allopregnanolone in humans is still needed. However, two clinical trials have been launched. A phase II clinical trial to test the safety and effectiveness of allopregnanolone in adults with moderate and severe TBI is ongoing (http://clinicaltrials.gov/ct2/show/NCT01673828?term=traumatic+brain+injury+and+allopregnanolone&rank=1 (2012). A phase I clinical trial to evaluate the safety and tolerability of allopregnanolone, in treating mild cognitive impairment and Alzheimer's disease is leaded by Prof. Brinton and Prof. Schneider.

For therapeutic development, a careful evaluation is needed. It is critical to choose the doses and treatment regimen that shows the best benefit/risk ratio and that promotes neuroprotection without over-exposure and with minimal adverse side effects. For example, it is important to avoid a too rapid withdrawal from progesterone, as it has been shown to exacerbate ischemic damage, and to increase excitotoxicity, anxiety, and susceptibility to seizure [179]. Concerning allopregnanolone, intermittent treatment regimens was neuroprotective in several neurodegenerative disease models [18,147,173,174,180], while continuous infusion worsen

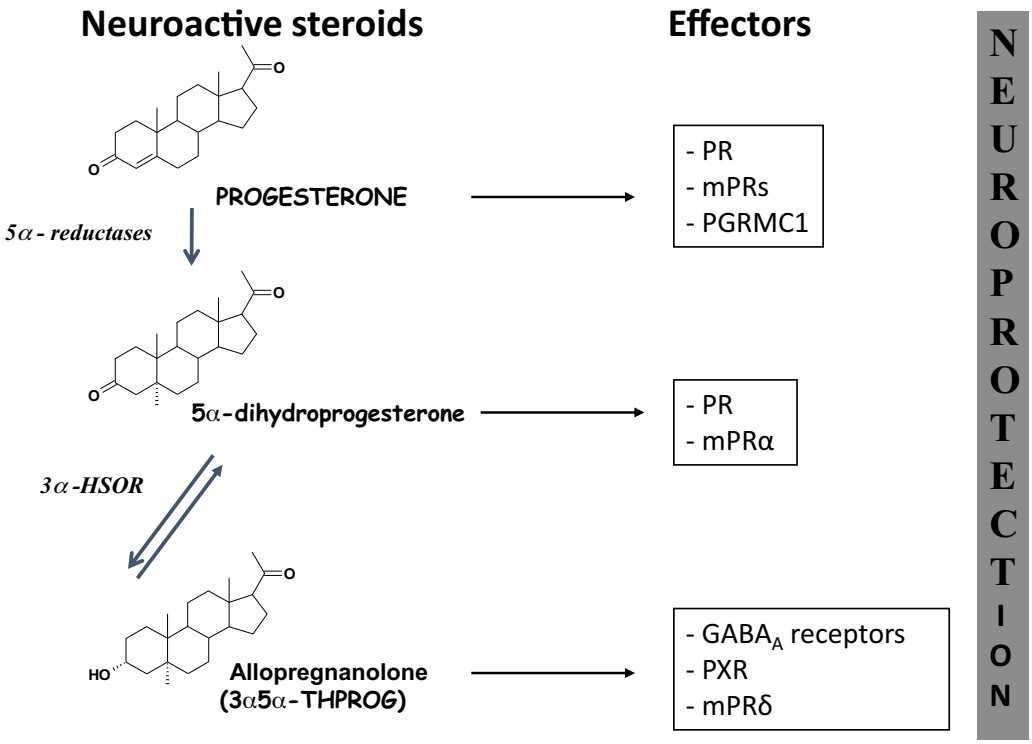

Fig. 3. Progesterone and allopregnanolone are pleotropic neuroprotective agents using different mechanisms of actions. Progesterone acts via genomic mechanisms after binding to the classical intracellular receptors (PR) or has rapid membrane actions via specific membrane receptors (mPR) or membrane binding sites (PGRMC1). Some effects of progesterone may be mediated by its metabolites. Progesterone is bio-converted to 5α-dihydroprogesterone, which binds to the classical receptors PR and has relatively high binding affinity for mPRα. 5α-dihydroprogesterone is converted to allopregnanolone (3α,5α-tetrahydroprogesterone) by the 3α-hydroxysteroid oxidoreductase (3α-HSOR) enzyme. Because of its hydroxyl group at C3, allopregnanolone has no affinity for the intracellular PR, but is a potent allosteric modulator of $GABA_A$ receptors. However, allopregnanolone can activate gene transcription via PR after being converted back to 5α-dihydroprogesterone. Some neuroprotective effects of allopregnanolone may be mediated by the pregnane X receptor (PXR) or by the membrane progesterone receptor mPRδ.

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outcome in a model of Alzheimer disease [181]. A recent study showed that allopregnanolone impairs the encoding and the consolidation of object memory [182], confirming and extending previous observations [183–185]. Allopregnanolone exerts anxiolytic effects however like all $GABA_A$ receptor agonist it may induce pronounced adverse effects like anxiety, irritability, aggressivity, seizure, increased pain in 3–8% of patients. These paradoxical effects may explain sex steroid induced negative mood symptoms in some persons [186]. These findings should be taken into consideration when evaluating the therapeutic potential of allopregnanolone.

Concerning the neuroprotective effects of progesterone, the prevailing view was that they may be mediated by its metabolite allopregnanolone, a potent modulator of $GABA_A$ receptors, We have now demonstrated a key role of the classical intracellular progesterone receptors (PR) in the neuroprotective effects of progesterone after stroke and spinal cord injury. The identification of PR as a neuroprotective drug target opens new perspectives for the use of synthetic progestins such as Nestorone, already validated for contraception and hormone replacement therapies, as neuroprotective agents. In addition to their selective actions, the high potency of some progestins may allow their administration at low doses. On the other hand, the choice of using natural progesterone instead of progestins could be a better option motivated by the larger range of mechanisms of actions of progesterone comparatively to progestins. For example, several progestins which are potent PR agonists do not bind to or activate mPRα [187] which mediates antiapoptotic actions of progesterone by an independent pathway [127].

When considering the use of synthetic progestins specifically designed to target the intracellular PR, it is important to be aware that they belong to different classes, and have different effects and pharmacological profiles [188,189]. Thus, many synthetic progestins have also an affinity to androgen and glucocorticoid receptors [177,190]. The binding of progestins to other receptors other than PR may generate undesirable side effects and adverse outcome. For example, the synthetic 17-OH progesterone derivative medroxyprogesterone acetate, which also activates androgen and glucocorticoid receptors, inhibits the effects of estradiol in the nervous system [190–194], is inefficient in promoting myelin repair [195], is harmful for neural cells [196] and increases disease severity in EAE experimental model [197]. The 19-norpregnane derivatives (promegestone, nomegestrol acetate and Nestorone) that bind almost exclusively to PR, will not affect immune system in the same manner as progestins which activate the glucocorticoid receptor [198]. Nestorone, a 19-norpregnane derivative, has been shown to have positive effects on neuroregeneration and repair of brain damage, as well as to promote myelin repair [22,195]. In addition to progestins having agonistic activity on PR, selective progesterone receptor modulators (SPRM), which display tissue-specific activities, deserve more attention [199]. Their potential neuroprotective benefits warrant further investigation.

Our results demonstrated the efficacy of allopregnanolone treatment in preventing neuronal death in experimental models of stroke and spinal cord injury by mechanisms independent of PR and rather involving $GABA_A$ receptors [22,23]. After spinal cord injury, there is an increase of glutamate release and a decrease of GABAergic tone leading to neuronal hyper excitability [200]. Thus, treatment with allopregnanolone or other molecules modulating $GABA_A$ receptors represent another therapeutic option that should also be evaluated for neuroprotection in stroke and spinal cord injury. Diazepam and chlormethiazole, GABA receptor agonists have been shown to be efficient in reducing infarct size and improving functional outcome in animal models of cerebral ischemia [201–203]. However, the sedation effects of GABA receptor agonists may limit their application in acute stroke patients due to the potential risk of stupor. Furthermore, sedation impairs cognitive function. A recent review of trials investigating the efficacy and safety of GABA receptor agonists does not provide evidence to use Diazepam or chlormethiazole for the treatment of patients with acute ischemic or hemorrhagic stroke [204]. Newer neuroprotective agents with greater efficacy than chlormethiazole should be evaluated. The side effects of $GABA_A$ agonists such as sedation, development of tolerance, consecutive abuse liability, and withdrawal symptoms may render their use problematic if a long-term treatment is needed, as for neurodegenerative diseases. In these cases, the use of the natural neurosteroid, allopregnanolone or drugs that increase its synthesis would be a preferable option.

Stroke, TBI, spinal cord injury and Alzheimer Disease have a high economic impact, as they are a major cause of death and neurological disabilities. For TBI, phase III clinical trials using progesterone are under evaluation. For Alzheimer disease, a phase I trial is ongoing. For spinal cord injury and stroke, trials using progesterone or allopregnanolone are lacking. However, to develop a safe and efficacious progesterone or allopregnanolone – based therapies providing neuroprotection, and stimulating endogenous repair mechanisms after stroke and spinal cord injury, more experimental data need to be generated. A better understanding of the mechanism of action of progesterone and selected progestins will help in defining better therapies.

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