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REVIEW PAPER

Steroids in Stroke with Special Reference to Progesterone

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Abstract
Both sex and steroid hormones are important to consider in human ischemic stroke and its experimental models. Stroke initiates a cascade of changes that lead to neural cell death, but also activates endogenous protective processes that counter the deleterious consequences of ischemia. Steroids may be part of these cerebroprotective processes. One option to provide cerebroprotection is to reinforce these intrinsic protective mechanisms. In the current review, we first summarize studies describing sex differences and the influence of steroid hormones in stroke. We then present and discuss our recent results concerning differential changes in endogenous steroid levels in the brains of male and female mice and the importance of progesterone receptors (PR) during the early phase after stroke. In the third part, we give an overview of experimental studies, including ours, that provide evidence for the pleiotropic beneficial effects of progesterone and its promising cerebroprotective potential in stroke. We also highlight the key role of PR signaling as well as potential additional mechanisms by which progesterone may provide cerebroprotection.

Keywords Cerebral ischemia · Sex differences · Progesterone receptors · Aging · Cerebroprotection · Neuroprotection

Introduction

Ischemic stroke is caused by the interruption of blood supply to brain tissue due to the occlusion of an artery by a thrombus, whereas the rupture of a brain artery leads to hemorrhagic stroke (Amarenco et al. 2009). Cerebral ischemia results in the rapid death of neural cells and in neurological deficits such as loss of some sensorimotor functions, paralysis, depression, and dementia. It is the leading cause of adult disability and the second leading cause of dementia and death in industrialized countries (Lo et al. 2003; Feigin et al. 2009).

Sex is a crucial parameter to consider when designing experiments to investigate the pathophysiology of stroke and to develop therapeutic pharmacological strategies. This aspect has been neglected for a long time, most studies having been carried out in male animals to avoid variability related to the female estrus cycle. However, recently, distinct pathophysiological mechanisms and different therapeutic responses according to sex have been described, notably those concerning brain functions (McCarthy et al. 2012). Incorporation of sex as a variable in the design of experiments is highly needed and recommended (http://www.nimh.nih.gov/researchfunding/scientific-meetings/2011/sex-differences-in-brain-behavior-mental-health-and-mental-disorders/index.shtml).

Several studies have shown that both sex and steroids are important factors to be taken into consideration when studying injury mechanisms and outcomes following brain ischemia (Cheng and Hurn 2010; Choleris et al. 2018; Herson and Hurn 2010). Recently, Liberale and colleagues have nicely reviewed and discussed the multiple sex differences in ischemic stroke (Liberale et al. 2018).

Sex Differences in Stroke

In humans, sex has an important impact on the etiology of stroke. Epidemiological studies have indeed revealed marked

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sex differences in stroke incidence, prevalence, ethiology, severity outcomes, and death (Reeves et al. 2008; Appelros et al. 2010; Haast et al. 2012; Wilson 2013; Gibson 2013). Stroke incidence and mortality rates are higher in men than in women, who are considered to be protected by their ovarian hormones (Petrea et al. 2009). Importantly, there exists an interaction between sex and age (Roy-O'Reilly and McCullough 2018). Whereas stroke rates increase with age in both sexes, starting after the age of 50, they become significantly higher in women when compared to men around the age of 80 (Petrea et al. 2009; Roger et al. 2011; Mozaffarian et al. 2016).

Experimental studies have demonstrated that sex is a key parameter for ischemic stroke outcomes. Transient or permanent middle cerebral artery occlusion (MCAO) with a filament is a commonly used model of ischemic stroke in rodents. Transient MCAO, followed by abrupt reperfusion, may be considered as a translational model of endovascular thrombectomy, which has become a reference therapy for patients with large vessel occlusion (Sutherland et al. 2016).

During their reproductive period, young female rats show smaller ischemic brain infarcts than aged-matched male rats after transient MCAO (Alkayed et al. 1998). However, this sex difference is no longer observed in females deprived of their ovarian steroids by ovariectomy and in aging senescent females (Alkayed et al. 1998, 2000). In another study, young female rats showed smaller infarcts and reduced sensorymotor deficits than young male rats or middle-aged female rats (Selvamani et al. 2014). Sex differences in the susceptibility to ischemia and its outcomes have been shown in different experimental models, of note even in the presence of comorbidities such as diabetes or hypertension (Toung et al. 2000; Carswell et al. 1999; Herson and Hurn 2010; Li et al. 2004).

Mechanisms underlying these sex differences include cell death pathways after ischemic stroke (Reeves et al. 2008; Yuan et al. 2009; Liu et al. 2011; Gibson 2013; Sohrabji et al. 2017; Choleris et al. 2018). In males, ischemic cell death is mainly the consequence of the activation of the poly (ADP ribose) polymerase 1 (PARP-1), a DNA repair enzyme involved in the caspase-independent pathway of apoptosis. The oxidative stress due to ischemia leads to the formation of single stranded DNA molecules, causing the activation of PARP-1. This activation induces cytosolic nicotinamide adenine dinucleotide (NAD) depletion, inhibition of glycolysis and depolarization of mitochondria and finally cell death (Alano et al. 2010). Conversely, in females, ischemic neural cell death mainly involves caspase-9 and caspase-3 activation. Thus, pharmacological inhibition of PARP-1 improved outcomes in males but not in females (Eliasson et al. 1997; McCullough et al. 2005), whereas caspase inhibition was beneficial in females but not in males (Liu et al. 2011). Interestingly, one study reported that deletion of PARP-1 increased ischemic damage in females but reduced infarct in males (Liu et al. 2011).

Sex Steroids and Stroke

In both women and men, age is the strongest risk factor for ischemic stroke, and the lifetime risk of stroke in both sexes shows a gradual increase from the age of 50 onwards (Petrea et al. 2009; Chen et al. 2010). Moreover, stroke mortality, morbidity and poor functional recovery are higher in the elderly. In women, stroke rates increase after menopause and become higher at an advanced age when compared with men (Petrea et al. 2009). The increase in stroke risk after menopause has been related to hormonal changes. Indeed, circulating levels of estradiol are reduced by more than 90% in women 5 years after menopause when compared to premenopausal women, and they even further decline thereafter (Rothman et al. 2011). However, it is important to note that postmenopausal women are not completely deprived of estrogens, as both estrone (about 40 pg/ml), its sulfated conjugate (about 250 pg/ml), testosterone (about 100 pg/ml), dehydroepiandrosterone (DHEA, about 2 ng/ml), and dehydroepiandrosterone sulfate (DHEAS, about 600 ng/ml) continue to circulate at significant levels in postmenopausal women as determined by liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Rothman et al. 2011; Wang et al. 2015; Martel et al. 2016). Estrone can be converted to estradiol by the 17ß-hydroxysteroid dehydrogenase, and androgens to estrogens by the aromatase, an enzyme abundant in brain, adipose tissues, bone and liver (Arevalo et al. 2015). Circulating DHEA and DHEAS, provided by the postmenopausal ovaries (about 20%) and adrenal glands, are a major precursor for the synthesis of biologically active steroid hormones inside hormone-sensitive tissues (Labrie 2015).

Increased stroke risk in women related to menopause has also been associated with the age-dependent increase in risk factors, including abdominal adiposity, increased levels of triglycerides and cholesterol, enhanced insulin resistance and elevated blood pressure (Lisabeth and Bushnell 2012). Interestingly, many of the stroke risk factors, including metabolic dysfunctions and a generalized proinflammatory milieu, are consequences of decreased ovarian functions (Della Torre et al. 2014). Androgens may increase blood pressure which is a major risk factor for stroke in men. Thus, in addition to the loss of female sex hormones, the increasing levels of testosterone with aging may contribute to the increase in blood pressure and to the greater risk of stroke in women after menopause (Reckelhoff 2001).

Over the past two decades, obesity rates increased (Ford et al. 2014; Ogden et al. 2015) and as consequence, the related disorders are also increasing. Obesity is a risk factor of different diseases including hypertension, sleep disorders,

atherosclerosis, cardiovascular diseases, stroke, and diabetes (Sharma et al. 2017). There is a sexual dimorphism with respect to adipose tissue deposition, distribution, and function; sex steroid hormones play a key role as endogenous regulators of adiposity (Palmer and Clegg 2015; White and Tchoukalova 2014). For example, estrogens protect against obesity by decreasing food intake and increasing energy expenditure in women. Men show more visceral adipose deposits which are positively correlated with growing cardiovascular risk. In contrast, females shows subcutaneous adipose deposits before menopause; this feature is associated with lower cardiovascular risk. After menopause, the decrease of estrogens leads to a shift in favor of the visceral fat, as seen in men, and an increase in cardiovascular risks and stroke (Palmer and Clegg 2015).

In addition to the systemic health factors, age-related changes in the brain contribute to its increased vulnerability to ischemic injury. They comprise multiple degenerative processes, structural changes in white matter, small-vessel diseases, reduced brain weight and intraneuronal inclusions of tau and α-synuclein (Chen et al. 2010).

In men, similar to women, the incidence of stroke gradually increases from the age of 50 onwards (Petrea et al. 2009). However, in contrasts to the ovaries, there is no abrupt decline in testicular activity at this age. Instead, testosterone levels gradually decrease in men from about 40 years onwards (Andersson et al. 2007; Huhtaniemi et al. 2012). However, it is important to note that individuals differ in terms of hormonal aging. Although the mean decrease in testosterone levels in men is gradual, the rate of decline is more important in some aging men than in others. The incidence of hypogonadal testosterone levels increases progressively: about 20% of men over 60 and about 50% of men over 80 years of age (Harman et al. 2001).

According to the prevailing consensus, ovarian estradiol and progesterone protect women against stroke, but the role of androgens in men is more controversial. As in the young adult population, men have a higher incidence of stroke and elevated levels of androgens have been suspected to represent a risk factor for stroke vulnerability. However, there is little evidence for this assumption, and the established link between anabolic steroid abuse and cardiovascular pathologies does not provide information on the role of endogenous androgens (Quillinan et al. 2014). On the other hand, the age-dependent increase in the incidence and severity of stoke in men suggests a protective effect of androgens (Quillinan et al. 2014). However, in spite of reported benefits of the therapeutic normalization of low testosterone levels (Sharma et al. 2015), concerns have been raised about the cardiovascular safety of testosterone therapy in aging men (Vigen et al. 2013). When discussing the usefulness of testosterone replacement, it is important to be aware that elevated levels of DHEA and DHEAS contribute to the pool of androgens and estrogens present in aging men, as they do in women (Labrie 2010).

Animal studies have provided strong evidence for cerebroprotective effects of steroid hormones after MCAO. We briefly review here the roles of estrogens and androgens. The effects of progesterone and its metabolites are discussed in detail in paragraph 4. The cerebroprotective effects of estrogens after an ischemic insult have been documented by a large number of studies and have been extensively reviewed (Wise et al. 2001; McCullough and Hurn 2003; Gibson et al. 2006; Herson et al. 2009; Lebesgue et al. 2009; Strom et al. 2009; Liu et al. 2010; Inagaki and Etgen 2013; Hurn and Macrae 2000). Treatment with either low or supraphysiological doses of estradiol has been shown to protect the female rat brain against stroke injury (Dubal et al. 1998; Suzuki et al. 2009; Carpenter et al. 2016). Importantly, protective effects of estrogen treatment are also observed in the presence of comorbidities such as diabetes (Toung et al. 2000).

A systematic meta-analysis has revealed that most experimental studies showing protective effects of estradiol after ischemic stroke used ovariectomized females. Some of the studies performed in gonadally intact young adult females failed to show beneficial effects of estradiol treatment, most likely because of the protective effects of endogenous ovarian hormones (Gibson et al. 2006). Cerebroprotective effects of endogenous ovarian hormones have been demonstrated by ovariectomy. Removing the ovaries of female rats indeed resulted in increased infarct volumes (Alkayed et al. 1998; Rusa et al. 1999; Inagaki and Etgen 2013). Larger infarcts were observed in cycling female rats in proestrus phase when their circulating levels of estrogens are low comparatively to metestrus (Carswell et al. 2000). Moreover, plasma estradiol levels have been shown to be inversely correlated with cortical infarct volume and neutrophil accumulation (Liao et al. 2001). These observations suggest an important neuroprotective role of endogenous estrogens. Interestingly, treatment of gonadally intact female mice with the intracellular estrogen receptor (ER) antagonist ICI182,780 increased ischemic infarct volumes, confirming the importance of endogenous estrogens and demonstrating the involvement of ER signaling (Sawada et al. 2000).

The ERα isoform, which is upregulated in response to MCAO, mediates the early cerebroprotective effects of estradiol. Thus, 24 h after cerebral ischemia, the neuroprotective effects of estradiol observed in wild-type mice were also observed in ERβ-KO mice but not in ERα-KO mice (Dubal et al. 2001). However, while ERα plays a critical role in the acute phase of stroke, both ERα and ERß are necessary for the stimulation of neurogenesis within the subventricular zone observed at 96 h after MCAO (Suzuki et al. 2007). Furthermore, a more recent study using a specific ERß agonist, reported a role of ERß in the recovery of sensorimotor

functions at later time points (8 and 17 days post-ischemia) (Madinier et al. 2014).

Effects of estrogens on the brain are age-dependent. Treatment of middle-aged ovariectomized female rats with physiological doses of estradiol decreased infarct volume by about 50% as it did in young ovariectomised female rats (Dubal and Wise 2001; Wise et al. 2001). This was an unexpected finding as at the middle age, the ability of estradiol to regulate the hypothalamo-pituitary axis has already markedly decreased (Downs and Wise 2009). With age, the neuroprotective efficacy of estrogens may decrease and estrogens may even exert adverse effects. Thus, in acyclic reproductive senescent female rats, estrogen treatment increased infarct volumes (Selvamani and Sohrabji 2010). As estradiol exerts its neuroprotective effects in synergy with IGF-1, the age-dependent decrease in IGF-1 levels may explain why estradiol ceases to be neuroprotective in aged females (Arevalo et al. 2015).

Estradiol treatment is also protective in male rats exposed to transient or permanent MCAO (Hawk et al. 1998; Toung et al. 1998; Perez-Alvarez et al. 2012). Different estrogen-mediated cerebroprotective mechanisms have been reported, including anti-inflammatory effects, protection against apoptosis, enhanced angiogenesis and increased neurogenesis (McCullough and Hurn 2003; Suzuki et al. 2009; Petrone et al. 2014).

As for the influence of testosterone on stroke in men, animal studies have revealed a complex picture, with androgens having either deleterious or protective effects (Quillinan et al. 2014). Most studies showed that gonadectomy of young adult male rats reduced infarct volume suggesting that endogenous androgens exacerbate stroke injury in males (Quillinan et al. 2014). For example, castration of male rats decreased ischemic brain damage after transient MCAO, whereas replacement with testosterone increased infarct size. In contrast to testosterone, estradiol treatment was protective (Hawk et al. 1998). Likewise, treatment of castrated male rats with the 5α-dihydrotestosterone (5α-DHT), a metabolite of testosterone that is not converted to estrogens but binds with high affinity to the intracellular androgen receptor (AR), restored infarct volumes to those of uncastrated males (Cheng et al. 2007). However, it was then shown that the effects of testosterone and 5α-DHT are dose-dependent. Whereas castrated male mice treated with low doses of either androgen had smaller infarct volumes, those treated with higher doses had larger infarcts than non-treated castrated mice.

The testosterone effects were AR-dependent, as they could be blocked with flutamide (Uchida et al. 2009). Taken together, these results suggest that endogenous testicular androgens may increase the susceptibility of the brain to ischemic damage and that the adverse effects of androgens may involve AR signaling.

It is important to better define the doses–responses relationships of androgens in stroke, to know precisely what is the optimal dose that provide protective response and at which dose there is a transition to damage effect. In future studies, it will be important to determine the type of the doses–responses curves: sigmoidal, U-shaped or inverse U-shaped. Several drugs tested for stroke therapy showed U-shaped doses–reponses curves (Calabrese 2008).

As noted above, in contrast to testosterone, estradiol treatment was found to be protective against ischemic injury in male rats (Hawk et al. 1998; Toung et al. 1998; Perez-Alvarez et al. 2012). However, whereas chronic treatment with the ER antagonist ICI182,780 of female mice exacerbated ischemic damage after MCAO, this treatment had no effects in males (Sawada et al. 2000). This suggests that endogenous estradiol may play a significant role in the resistance of the brain to ischemic damage in females but not in males.

The absence of protective effects of elevated doses of testosterone and endogenous estrogens in males may come as a surprise. Indeed, conversion of testosterone to estradiol in the male brain by the aromatase enzyme is neuroprotective and plays a key role in the resistance of neural cells to a variety of insults (Garcia-Segura et al. 2003; Arevalo et al. 2015). However, we have to be aware that expression of the brain aromatase is upregulated in response to injury, mainly in astrocytes, which do not constitutively express the enzyme in adult rats (Garcia-Segura et al. 1999). The aromatase is also induced by MCAO in astrocytes of the peri-infarct area. This Increase was transient as it was observed at 24 h and 8 days, but neither at 2 h or at 30 days post-ischemia (Carswell et al. 2005). These observations suggest a delay in the actions of testosterone-derived estradiol, which may regulate neuroinflammation and play a role in regenerative processes. The importance of aromatase is also suggested by the observation that MCAO-induced neurogenesis is reduced in aromatase knockout mice (Li et al. 2011).

Sex-Dependent Changes in Endogenous Steroid Levels in Response to Stroke

Increase in steroid levels is part of endogenous mechanisms triggered after ischemic stroke. In a first study, we have shown that levels of progesterone and 5α-dihydroprogesterone (5α-DHP) were highly upregulated in the brain of male mice as early as 6 h after MCAO (Liu et al. 2012). We have then performed a detailed study to investigate the temporal changes of steroid levels in brain and plasma of both male and female mice at diestrus phase using gas chromatography-tandem mass spectrometry (GC-MS/MS) (Zhu et al. 2017). Our study revealed marked differences in brain steroid levels between males and females in the absence of injury and also

sex-dependent changes in endogenous steroid levels after MCAO. Data are summarized in Fig. 1.

Stroke Increased Brain Levels of Progesterone and its Neuroactive 5α-Reduced Metabolite in Male But Not in Female Mice

Surprisingly, levels of 5α-DHP, a natural PR agonist (Ruprecht et al. 1993), were higher in male than in female brain of intact mice. After MCAO, brain progesterone and 5α-DHP levels were rapidly upregulated in males, but not in females, reaching highest levels at 6-h post-MCAO (Zhu et al. 2017). Levels of the potent $GABA_A$ receptor active progesterone metabolite 3α,5α-tetrahydroprogesterone (3α,5α-THP, allopregnanolone) were also higher in the male brain, but its levels did not significantly change with time after MCAO (Fig. 1a) (Zhu et al. 2017).

Stroke Increased Levels of Glucocorticoids in Both Male and Female Mice

Levels of steroids that are related to stress, namely, deoxycorticosterone (DOC), 5α-dihydrodeoxycorticosterone (5α-DHDOC), 3α,5α-tetrahydrodeoxycorticosterone (3α,5α-THDOC) and corticosterone were also upregulated in response to ischemia in both plasma and brain of males and females. However, in contrast to progesterone and its metabolites, levels of corticosterone remained lower in brain than in plasma between 1- and 4-h post-MCAO. Interestingly, a marked sex difference was observed at 6 h: in males, brain levels of corticosterone were highly increased, reaching plasma levels (about 200 ng/g tissue), whereas in females, brain levels remained significantly lower than in plasma (about 70 ng/g tissue vs 190 ng/ml in plasma) (Zhu et al. 2017) (Fig. 1b). Therefore, after ischemic injury, the male brain is transiently exposed to higher amounts of corticosterone than the female brain. Glucocorticoids and stress are known to aggravate ischemic brain damage (Sapolsky and Pulsinelli 1985; Sugo et al. 2002). It is thus conceivable that the increase in levels of progesterone and its 5α-reduced metabolite in the male brain may be a mechanism to protect neural cells against the damaging effects of elevated glucocorticoid levels. Consistent with this hypothesis, a recent study has shown that chronic stress exacerbated inflammation and neural loss in the hippocampus of male rats after global ischemia and that progesterone was efficient in decreasing the deleterious effects of stress (Espinosa-Garcia et al. 2017).

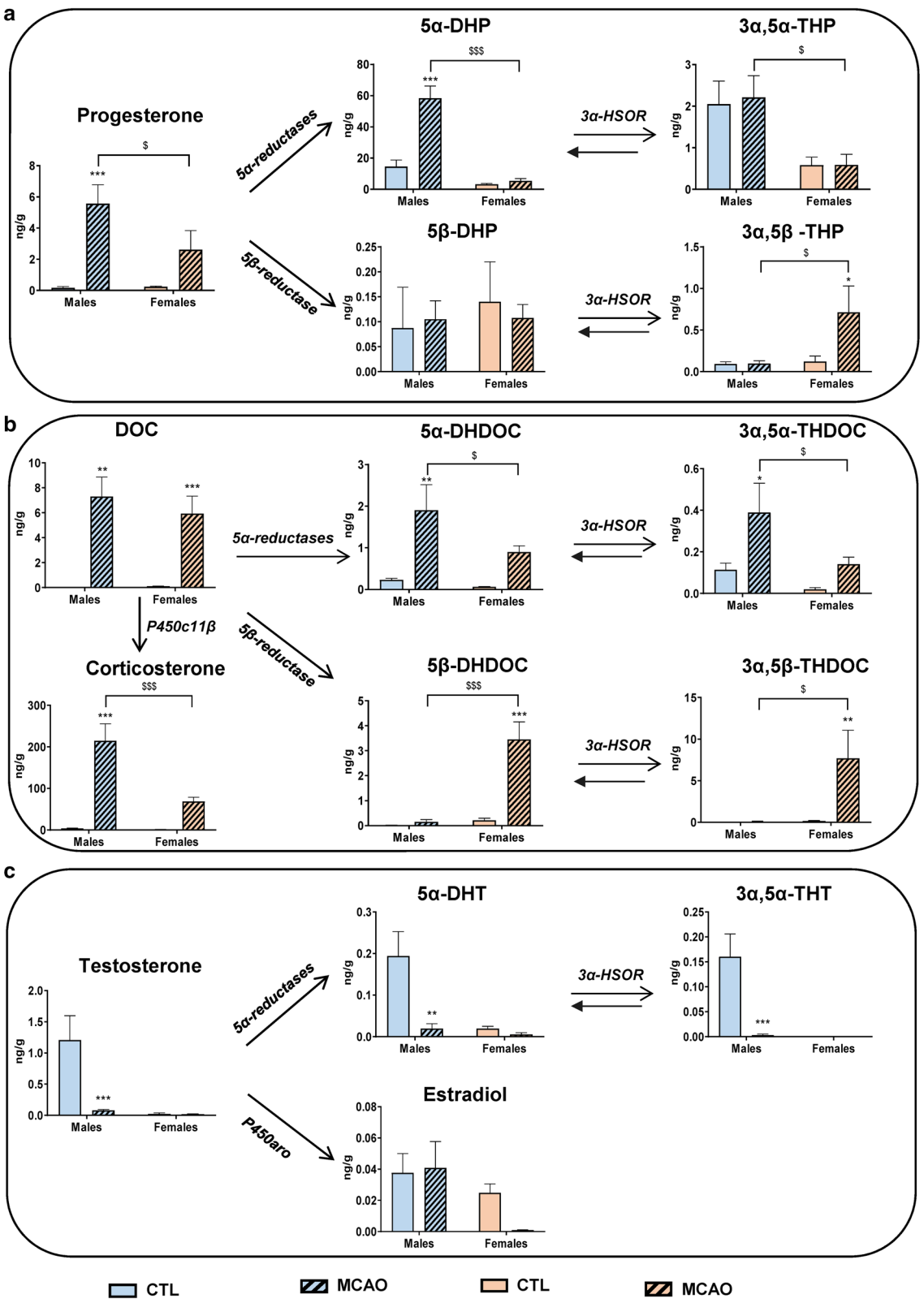
Stroke Decreased Levels of Androgens in Males and had no Significant Effects on Estradiol Levels

In contrast to progesterone and glucocorticoids, brain levels of testosterone and 5α-DHT decreased as early as 1-h post-MCAO in males. At 6 h, their brain levels were respectively 15- and 10-times lower than in intact male mice (Fig. 1c). The downregulation of androgens may be the consequence of a disruption of the hypothalamo-pituitary-gonadal axis, or of the stress caused by stroke. Indeed, stress, adrenal hyperactivity and high doses of corticosteroids impair different aspects of reproduction including steroidogenesis (Rivest and Rivier 1991; Tilbrook et al. 2000; Orr et al. 1994; Maric et al. 1996; Kostic et al. 1998). Another possibility may be a competitive inhibition of the P450c17 enzyme necessary for testosterone synthesis, by the increased levels of progesterone and 5α-DHP as they are also substrates of this enzyme (Shet et al. 1994; Auchus et al. 2003).

Brain levels of estradiol were low in intact mice and did not differ between males and females. In both sexes, no significant changes in brain estradiol were observed between 1 and 24 h after MCAO, although there was a tendency to decreased estradiol levels in females at 6-h post-MCAO (Zhu et al. 2017) (Fig. 1c). However, it is important to note that in our study steroid levels were measured in large brain samples (the whole ischemic hemisphere); therefore, localized changes in steroid levels in specific brain regions after MCAO cannot be excluded. For instance, levels of 17β-estradiol have been shown to increase after MCAO in the dialysate from parabrachial nucleus of male rats. This increase was transitory with a maximum at 10 min followed by a decrease to levels bellow baseline by 90-min post-MCAO (Saleh et al. 2004). Furthermore, the same group showed a continuous increase of 17β-estradiol in the dialysate from the central nucleus of the amygdala beginning at 30-min post-MCAO with maximal values measured at 4-h post-MCAO (Saleh et al. 2005).

Stroke Increased Levels of 5ß-Reduced Steroids in Female But Not in Male Mice

In contrast to what was observed in males (increase of 5α-reduced steroids following MCAO), an increase of 5β-reduced steroids was observed in the brain of female mice. In particular, brain levels of 3α,5β-THP, 5β-DHDOC, and 3α,5β-THDOC increased in females and were higher than in males at 6-h post-MCAO (Fig. 1a, b) (Zhu et al. 2017). This observation may be of importance, as 3α,5β-THP and 3α,5β-THDOC are, positive allosteric modulators of $GABA_A$ receptors. 5β-reduction of steroids



may also be a mechanism to regulate their concentrations and their availability for receptors (Belelli et al. 1996; Chen and Penning 2014; Gunn et al. 2015).

◂**Fig. 1** Brain levels of steroids in male and female mice intact and 6 h post-MCAO, as analyzed by gas chromatography tandem mass spectrometry (GC/MS/MS). Young male and female mice (diestrus) were subjected to 1 h MCAO and brain steroid levels were measured at 6-h post-MCAO. Data are expressed as means ± SEM (ng/g of tissue; $n = 6$–10 per group) and were analyzed by two-way ANOVA (surgery × sex) followed by Newman–Keuls multiple comparisons tests. $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$ MCAO versus intact; $^{\$\$\$}p < 0.001$, $^{\$}p < 0.05$ MCAO females versus MCAO males as indicated. *DHP* dihydroprogesterone, *THP* tetrahydroprogesterone, *DOC* 11-deoxycorticosterone, *DHDOC* dihydro-11-deoxycorticosterone, *THDOC* tetrahydro-11-deoxycorticosterone, *DHT* dihydrotestosterone, *THT* tetrahydrotestosterone, *3α-HSOR* 3α-hydroxysteroid oxidoreductase, *P450aro* P450 aromatase (data from Zhu et al. 2017)

Progesterone Receptor Signaling Mediates the Early Endogenous Cerebroprotection After Ischemic Stroke in Young and Aging Male and Female Mice

If the endogenous progesterone and 5α-DHP are important for the cerebroprotection at the acute phase of stroke, PR may be a key mediator. To test this hypothesis, we studied the response of PR Knockout mice to ischemia.

We first used available PR knockout mice (PRKO) lacking PR expression in all tissues (Ismail et al. 2002). We demonstrated in young adult male mice that lack of PR expression increased ischemic brain infarct and motor dysfunctions at 6 and 24 h, but not at 48 h post-MCAO (Liu et al. 2012). These observations highlight the importance of PR-dependent mechanisms in the protection of the brain at the acute phase after stroke. To go further, we generated a new transgenic mice line (PR^{NesCre}) selectively lacking PR expression in neural cells, to evaluate the relative role of PR specifically expressed in the brain. At 6-h post-MCAO, both young and aging male and female PR^{NesCre} mice showed exacerbated neurological deficits and increased infarct volumes comparatively to their control $PR^{loxP/loxP}$ littermates that express normal levels of PR (Fig. 2) (Zhu et al. 2017).

However, the invalidation of PR expression had more deleterious effects in young males than in young females. In particular, the exacerbation of tissue damage in PR^{NesCre} mice was more pronounced in males than in females (Fig. 2b) (Zhu et al. 2017). This observation points to additional endogenous processes, independent from neural PR signaling, which contribute to the prevention of tissue loss in the female brain. These additional mechanisms may depend on ERα as its expression is up-regulated early after cerebral ischemia in females (Dubal et al. 2006) but not in males (Westberry et al. 2008).

Our findings demonstrate an early endogenous cerebroprotective mechanism depending on PR function in neural cells in young and aging males and females. This strongly suggests that selective ligands of PR may be useful for cerebroprotection after ischemic stroke. However, they may show different efficiencies in young males and females.

Progesterone is a Promising Pleiotropic Cerebroprotective Agent After Stroke

Neural cells are very sensitive to oxygen and glucose deprivation, and they rapidly start dying after ischemic stroke. The major problem is the progressive spreading of nervous tissue damage. There is thus an urgent need for cerebroprotective agents that limit the death of neurons in the peri-infarct area (Stankowski and Gupta 2011).

Experimental models of ischemic stroke have provided strong evidence for the cerebroprotective effects of progesterone (Gibson et al. 2009; Wong et al. 2013b). The majority of studies that evaluated the effects of progesterone treatment on infarct size and functional outcomes reported beneficial effects. Only few studies reported no effects and one study reported a deleterious effect (Gibson et al. 2009; Wong et al. 2013b). For the studies showing beneficial effects, progesterone was administrated at moderate doses early after ischemia. In contrast, administration of very high doses of progesterone before ischemia induction to ovariectomised females showed no effect on cortical infarct and even an increase of the sub-cortical infarct when the treatment was chronic (Murphy et al. 2000). The observed negative results may be due to the high doses of progesterone, the time of treatment initiation, the endocrine status of animals at the time of ischemia and/or the early time of analysis. Therefore, endogenous progesterone levels, the dose, time, and schedule of progesterone treatment are all very important to be taken into account when designing preclinical studies.

The systematic meta-analysis by Wong et al. showed that in mice and rats exposed to either transient or permanent MCAO, progesterone treatment at moderate doses reduced infarct volume and improved functional recovery, such as the ability to remain on the rotarod and the reduction of neurological score deficits (Wong et al. 2013b). Most studies were performed in young males, some in ovariectomized young females and few in aging animals. Unfortunately, no study has been performed in gonadally intact young females (Wong et al. 2013a). The few studies that have used aged males, reported positive neuroprotective effects of progesterone (Wang et al. 2010; Yousuf et al. 2014a, b; Wali et al. 2014, 2016). Only three studies evaluated the effect of progesterone treatment in aging females. While one study showed a reduction of cortical infarct volume in aging female rats with chronic pretreatment by progesterone implants for 1 week (Alkayed et al. 2000); a second study showed no effect on infarct volume by acute treatment of progesterone initiated just 0.5 h before MCAO (Toung et al. 2004). A third study showed a reduction of

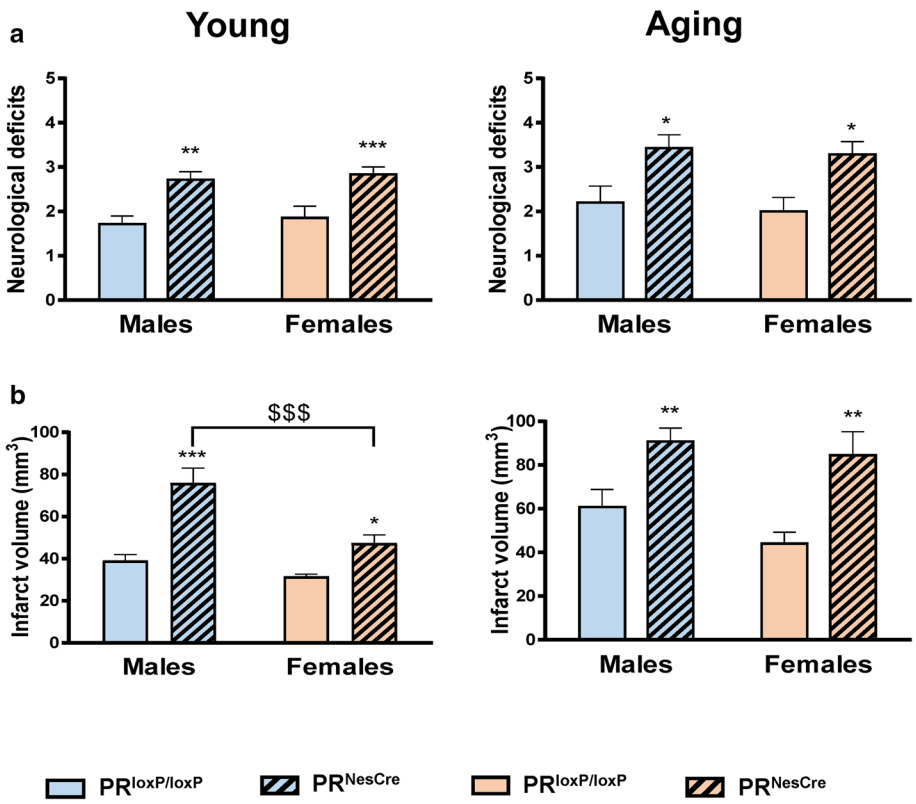


Fig. 2 Specific deletion of PR in neural cells leads to increased neurological deficits (**a**) and infarct volumes (**b**) in both young and aging male and female mice. Mice were subjected to 1 h MCAO and neurological deficit scores (higher scores reflect higher disability) and total infarct volumes were analyzed at 6-h post-MCAO. $PR^{loxP/loxP}$ mice: transgenic mice in which exon 2 of PR was flanked by loxP sites; mice expressing normal levels of PR. PR^{NesCre} mice: transgenic mice that selectively lack PR in the neural cells using the Cre-lox strategy (data from Zhu et al. 2017)

total infarct volume, but no effect on neurological scores when progesterone was administered at 1-, 6-, and 24-h post-MCAO (Gibson et al. 2011). Most studies except few ones used healthy mice and rats without any comorbidities or risk factors (Ankolekar et al. 2012). Studies using male hypertensive animals have shown beneficial effects of progesterone on infarct size and neurological outcomes at 7 and 14 days post-MCAO (Kumon et al. 2000; Wong et al. 2014; Yousuf et al. 2016). However, one study showed no effect of progesterone on lesion volume nor on neurological outcomes at 24-h post-MCAO in spontaneously hypertensive male rats (Spratt et al. 2014).

Treatment Schedule, Dose–Response, Time Window, and Mode of Progesterone Administration

The majority of experimental studies evaluating the effects of progesterone treatment in rodents used the dose of 8 mg/kg administered by subcutaneous and/or intraperitoneal injections (Wong et al. 2014; Gibson and Murphy 2004; Lee et al. 2015; Liu et al. 2012; Dang et al. 2011; Yousuf et al. 2016; Spratt et al. 2014; Sayeed et al. 2006, 2007; Ishrat et al. 2010, 2012; Wang et al. 2011; Gibson et al. 2011). The schedule of administration at 1-, 6-, and 24-h post-MCAO is the one that has been the most often used (Gibson et al. 2008; Wong et al. 2013b). Dose–response studies have also been performed (Chen et al. 1999; Wali et al. 2014, 2016; Yousuf et al. 2014a). The dose of progesterone with an optimal neuroprotective effect was 8 mg/kg. For instance, in a transient MCAO model, administration of progesterone at 2-h post-MCAO at the dose of 8 mg/kg reduced infarct size and improved functional outcomes, whereas treatment with 4-or 32-mg/kg had no effects (Chen et al. 1999). In a permanent stroke model, Wali and colleagues showed that moderate doses of progesterone (8- or 16-mg/kg) were efficient in reducing infarct volume and improving functional outcomes for up to 3 weeks of post-MCAO in aging rats. However, the dose of 8 mg/kg was more efficient in improving the spatial memory. Of note, progesterone treatment still provide neuroprotection when treatment was initiated at 6-h post-MCAO (Wali et al. 2014). In a more recent study, the same group showed that the beneficial effects of progesterone treatment on infarct size and neurological outcomes still be observed for up to 8 weeks post-MCAO (Wali et al. 2016).

One of the STAIR's recommendations is to test different modes of administration of therapeutic drugs in preclinical studies. We are currently investigating the cerebroprotective potential of intranasal administration of progesterone. We have shown that progesterone dissolved in oleogel and administrated intranasally penetrated efficiently into the brain, and is cerebroprotective in male mice subjected to MCAO. Furthermore, brain levels of corticosterone were lower in progesterone-treated mice than in vehicle mice, suggesting that this mode of administration is a non-stressful

route of progesterone delivery to brain that warrant evaluation in future experimental studies (Frechou et al. 2015; Guennoun et al. 2018).

Pleiotropic Effects of Progesterone Treatment After Ischemic Injury

As presented above, several experimental studies have shown that progesterone treatment decreases the extent of ischemic infarction and improves functional outcomes. The underlying mechanisms of progesterone effects are beginning to be unraveled. Thus, progesterone treatment has been shown to regulate different cellular and functional events important for cerebroprotection, including edema formation, neurotoxicity, blood–brain barrier (BBB) disruption, apoptosis, inflammatory responses and mitochondrial functions.

Progesterone Treatment Decreases Blood–Brain Barrier Disruption and Edema

One of the deleterious consequences of cerebral ischemia is the dysfunction of the blood–brain barrier (BBB) (Jiang et al. 2018). Excessive production of free radicals that cause oxidative stress activate matrix metalloproteases (MMPs). This activation leads to the degradation of the basal lamina as well as intercellular junctions of the BBB (Gidday et al. 2005; Yang et al. 2007). Infiltration of leukocytes contributes to the alteration of the BBB (Jiang et al. 2018; Kebir et al. 2007; McColl et al. 2007). BBB leakage results in edema formation, hemorrhagic transformation, and increased inflammation.

Different studies showed that progesterone limits BBB leakage after stroke. Thus, we have recently shown that intranasal administration of progesterone at the time of reperfusion attenuates BBB opening at 4-h post-MCAO (Frechou et al. 2015). This effect on the BBB during the early phase after stroke may contribute to the beneficial effects of progesterone on neuronal survival and on functional outcome observed at later time points. Likewise, Ishrat and colleagues demonstrated that progesterone treatment decreased the permeability of the BBB barrier at 72 h after ischemia by acting on the expression of MMPs, the pro-inflammatory molecules TNF-α and interleukin-6, and the tight junction proteins occludin 1 and claudin 5 (Ishrat et al. 2010). The effects of progesterone on BBB permeability and on the expression of the tight junction proteins were also demonstrated in vitro using mouse brain endothelial cells treated with thrombin (Hun Lee et al. 2015). Furthermore, progesterone decreased the hemorrhagic transformation, brain swelling, BBB leakage, and the induction of MMP-9 and VEGF expression observed in rats treated with tissue plasminogen activator (tPA) at 4.5-h post-MCAO (Won et al. 2014).

Cerebral edema is a major complication of ischemic stroke that contributes to increased mortality. Progesterone treatment has been found to be efficient in reducing brain edema after ischemic stroke (Grossman et al. 2004; Gibson et al. 2005; Liu et al. 2012; Jiang et al. 2016). Progesterone was also able to counter the increased edema formation induced by t-PA treatment after transient MCAO (Won et al. 2014).

Progesterone Treatment Decreases the Inflammatory Response

After cerebral ischemia, there is an acute and prolonged inflammatory response consisting of the early activation of microglia and astrocytes, the synthesis and release of pro-inflammatory cytokines and chemokines and the infiltration into the brain parenchyme of neutrophils, T cells, and macrophages. This cascade of events participates in brain tissue loss (Jin et al. 2010; Iadecola and Anrather 2011). There is a double function of microglia after stroke. Activated microglia can exert either beneficial or detrimental effects, depending on their phenotype (Hu et al. 2015; Ransohoff 2016; Ma et al. 2017).

Progesterone treatment has been shown to regulate the density and polarisation of microglia and to reduce pro-inflammatory cytokines and nitric oxide synthase-2 (Grossman et al. 2004; Habib and Beyer 2015; Ishrat et al. 2010; Jiang et al. 2009; Habib et al. 2014a, b; Coughlan et al. 2005; Won et al. 2015; Allen et al. 2016; Lammerding et al. 2016; Yousuf et al. 2016). Recently, Espinosa-Garcia et al. evaluated the anti-inflammatory potential of progesterone in the hippocampus of mice exposed to stress followed by global ischemia. They showed that stress exacerbated the inflammatory response by increasing the activation of microglia, affecting their phenotype, enhancing the expression of inflammatory cytokines, and reducing the expression of protective factors. Progesterone treatment counteracted the effects of stress and ischemia by mitigating the inflammatory response and regulating the polarization of microglia (Espinosa-Garcia et al. 2017). Inflammasomes are multiprotein complexes that play a key role in central nervous system inflammation and their activation represents a critical step in the neuro-inflammatory responses (Singhal et al. 2014). Recent studies showed that the anti-inflammatory effects of progesterone involve interactions between inflammasomes activation and their related regulatory miRNAs (Slowik and Beyer 2015).

Progesterone Treatment Reduces Brain Mitochondrial Dysfunction and Oxidative Damage

Mitochondria are the site of energy production and are major regulators of oxidative stress (Gaignard et al. 2018). Due to

the high metabolic rate and the low energy storage capacity in neurons, mitochondria play a key role in brain function. We have recently investigated the role of endogenous steroids in the brain mitochondria function (Gaignard et al. 2015). In particular, we have shown that mitochondrial respiration is higher, while oxidative stress is lower in the brain of young adult females as compared to young adult males. These differences were not observed in ovariectomised mice and in aged senescent mice (Gaignard et al. 2015). Our findings suggest that endogenous ovarian steroids may influence brain mitochondrial functions under physiological conditions.

With regard to stroke, mitochondria play a key role since they regulate energy production, oxidative stress, and cell death (Kalogeris et al. 2014; Gaignard et al. 2018). The drop in blood supply causes a decrease in ATP synthesis and the lack of oxygen causes a depolarisation of the inner mitochondrial membrane, leading to the production of high levels of reactive oxygen species (ROS). Energy drop and oxidative stress result in disturbance of ionic pumps and excitotoxicity, leading to cell death (Sims and Muyderman 2010; Abramov et al. 2007; Manzanero et al. 2013; Dirnagl et al. 1999). Mitochondria are thus promising therapeutic targets for promoting recovery from stroke (Jin et al. 2016).

Treatment with progesterone increased the level of the antioxidant enzymes such as superoxide dismutase, glutathione peroxidase and catalase, restored levels of total glutathione, and attenuated lipid peroxidase (Aggarwal et al. 2008; Ozacmak and Sayan 2009). Progesterone was also shown to inhibit the translocation of the apoptotic factor, cytochrome c from mitochondria to cytosol (Sayeed et al. 2009).

In a recent study, we have investigated the effects of progesterone on the brain mitochondrial respiratory chain and oxidative damage at 6-h post-MCAO (Gaignard et al. 2016). We observed a sex difference in stroke effects on the brain mitochondrial respiratory chain. The reduced flavin adenine dinucleotide ($FADH_2$)-linked respiration and the activity of complex II (CII) decreased in females but not in males. The reduced nicotinamide adenine dinucleotide (NADH)-linked respiration decreased in both males and females. The mitochondrial pool of reduced glutathione (GSH) is the main anti-oxidant factor and its levels regulate neuronal cell death (Wullner et al. 1999). As demonstrated by others (Anderson and Sims 2002), we showed that levels of mitochondrial GSH decreased after MCAO. We demonstrated that progesterone treatment is efficient in preserving mitochondrial functions that are altered by ischemia. Our findings identify the mitochondria as target of progesterone action after stroke and suggest that the effects of progesterone on mitochondrial function may be one of the mechanisms by which progesterone provide neuroprotection (Gaignard et al. 2016). Recently, Andarabi and colleagues provided further evidence for the beneficial effects of progesterone on brain mitochondrial function after ischemic injury (Andrabi et al. 2017). They indeed showed that progesterone (1) restored the function of mitochondrial respiratory chain by increasing the activities of complex I and complex II and the levels of complex V; (2) modulated different oxidative stress parameters such as lipid peroxidation, ROS production, and mitochondrial GSH; and (3) reduced the swelling of mitochondria, and the release of cytochrome c from mitochondria in the cytosol (Andrabi et al. 2017).

Progesterone Treatment Regulates Levels of Serotonin and Dopamine and Some Markers of Neurotoxicity and Neuroprotection

Neurotransmitter imbalance causes dysregulation of brain functions and may lead to secondary neuronal damage (Chen et al. 2014). Levels of dopamine and serotonin increased in the frontal cortex after ischemia and progesterone treatment counteracted this increase (Andrabi et al. 2017). Likewise, similar effects of ischemia and progesterone were observed for the activities of the monoamine oxidase and the acetylcholine esterase enzymes. Furthermore, progesterone was efficient in re-establishing the activity of the Na^+, K^+-ATPase that was decreased by ischemia thereby attenuating the mitochondrial damage (Andrabi et al. 2017).

Brain-derived neurotrophic factor (BDNF) and vascular endothelial growth factor (VEGF) regulate neuronal survival, neurogenesis and also vascular remodeling, angiogenesis, and brain plasticity (Greenberg et al. 2009; Jin et al. 2002; Ruan et al. 2015). BDNF is neuroprotective after cerebral ischemia (Chen et al. 2013), while VEGF may play a dual role. For instance, a delayed treatment with VEGF increased angiogenesis and improved functional outcomes. In contrast, treatment at the first hour following stroke leads to increased BBB permeability, hemorrhagic transformation and tissue damage (Zhang et al. 2000). Progesterone administration increased the levels of BDNF and VEGF in the peri-infarct at 72 h and decreased them at 14 days post-ischemia (Ishrat et al. 2012). In this study, progesterone was also shown to reduce apoptosis and its related proteins. Similarly, a recent study showed that progesterone decreased VEGF and increased BDNF levels in the cortex at day 3 post-MCAO. Progesterone increased neurogenesis in the sub-ventricular zone and the density of the newly generated neurons in the peri-infarct at day 7 post-MCAO. These effects could partially underlie the improvement of neurologic functions observed on days 7 and 14 post-MCAO (Jiang et al. 2016).

Modes of Action of Progesterone After Stroke: A Key Role of PR

The classical mechanism of action of progesterone is the regulation of gene transcription after binding to its intracellular receptors PR. Progesterone actions may also be mediated by specific membrane receptors, either the progesterone receptor membrane component 1 (PGRMC1) or the seven-transmembrane G protein-coupled progesterone receptors (mPRs). Progesterone is also a competitive inhibitor of sigma-1 receptors. Finally, progesterone may be converted to allopregnanolone, a potent modulator of $GABA_A$ receptors. All these mechanisms may contribute to the cerebroprotective actions of progesterone as all these receptors are largely distributed in the brain and as progesterone is actively metabolized to allopregnanolone in the brain (Schumacher et al. 2007; Guennoun et al. 2015).

As discussed in paragraph 3, our recent studies demonstrated a key role of PR in the endogenous cerebroprotection at 6- and 24-h post-MCAO (Liu et al. 2012; Zhu et al. 2017). PR is a limiting factor, as even heterozygous $PR^{+/-}$ mice showed larger ischemic infarcts comparatively to wild-type $PR^{+/+}$ mice (Liu et al. 2012). The key role of PR was also demonstrated after progesterone treatment. Indeed, progesterone treatment was efficient for decreasing infarct volume, neurological and motor deficits in wild-type $PR^{+/+}$ mice, but not in $PR^{-/-}$ knockout mice (Liu et al. 2012). Another study confirmed the importance of PR in stroke as progesterone decreased the infarct volume at 48-h post-MCAO in wild-type $PR^{+/+}$ but not in heterozygous $PR^{+/-}$ mice (Lee et al. 2015). To know if the activation of PR is sufficient to provide cerebroprotection, we used Nestorone: a potent and selective PR agonist with no unwanted interaction with other receptors (Kumar et al. 2000) and which is not converted to $GABA_A$ receptor-active metabolites (Kumar et al. 2017). We showed that Nestorone at a very low dose (100-times lower than progesterone) decreased infarct volume and motor deficits (Liu et al. 2012). We have recently extended our analysis concerning the role of PR. We have shown in particular that progesterone treatment (1) increased the density of neurons, of oligodendrocytes and of their precursors; (2) decreased the density of activated microglia and of astrocytes and of aquaporin 4 expression; and (3) that the selective invalidation of PR expression in neural cells blocked all these effects (Zhu et al. 2018).

Treatment with allopregnanolone has also been shown to be neuroprotective after MCAO. In particular, administration of allopregnanolone has been shown to decrease infarct volume, edema, motor deficits, BBB dysfunctions, neuroinflammation and the activation of the mitochondrial permeability transition pore (Sayeed et al. 2006, 2009; Ishrat et al. 2010; Liu et al. 2012). Although both progesterone and allopregnanolone are neuroprotective when administered after ischemic injury, their mechanisms of action are different. Allopregnanolone exerts cerebroprotective effects via PR-independent signaling pathway as it has no affinity for PR and its effects have also been demonstrated in PR knockout mice (Liu et al. 2012). As a positive modulator of $GABA_A$ receptor, allopregnanolone may counteract excitotoxic mechanisms by potentiating $GABA_A$ receptor-dependent decrease in neuronal excitability. Intracellular PR play a key role in mediating the effects of progesterone. Indeed, neuroprotective effects of progesterone are no longer observed in PR knockout mice and Nestorone, the selective PR agonist, is sufficient to provide efficient cerebroprotection at a very low dose. These findings also indicated that the in vivo bioconversion of progesterone to allopregnanolone is not the mechanism through which progesterone provides cerebroprotection, otherwise progesterone treatment would have been protective in PR knockout mice.

Although there is strong evidence for a key role of PR-depending signals in the mediation of the cerebroprotective effects progesterone, these findings do not exclude the involvement of additional progesterone signaling mechanisms, depending on the dose and timing of progesterone administration, and on the time and type of outcome measures. Cai et al. investigated the potential mechanisms underlying the neuroprotective effects of progesterone. They showed in particular that PR, via activation of the Src-ERK1/2 cascade, mediated the cerebroprotective effects of progesterone observed in the hippocampus at 48-h post-MCAO. In contrast, the acute protective effects observed at 1 h involved the antagonistic actions of progesterone on sigma-1 receptors, resulting in an attenuation of the NMDA-induced increase in intracellular calcium concentrations (Cai et al. 2008).

The phosphoinositide 3-kinase/protein kinase B (PI3K/Akt) cascade is a signal transduction pathway that regulates inflammation, cell survival in response to growth factors and is involved in cerebroprotection after ischemic stroke (Brazil et al. 2004; Zhao et al. 2006; Xu et al. 2008; Wang et al. 2009). The hypothesis that this pathway may mediate some of the beneficial effects of progesterone has been tested (Ishrat et al. 2012). Inhibiting the PI3K/Akt pathway with Wortmannin decreased the beneficial effects of progesterone on infarct size, edema, apoptosis and VEGF levels observed at 24-h post-MCAO (Ishrat et al. 2012).

Summary and Conclusions

Understanding the significance of sex differences in response to ischemic stroke injury and in cerebroprotection is fundamental for developing and refining effective treatment strategies that are beneficial for both men and women. Rather than ignoring sex and steroid hormones as variables, as done in

almost all preclinical studies, there is much to be gained by taken them into account for studying pathophysiological and therapeutic mechanisms.

Advances in the understanding the mechanisms of action of steroids in stroke will permit to envisage therapeutic approaches based on a combination of steroids or new molecules modulating their synthesis, their receptors or their signaling pathways. In particular, cerebroprotection by progesterone or molecules targeting PR signaling offer great promises for the treatment of stroke patients. Taking into account the steroid status of patients and reinforcing progesterone signaling should be exploited in therapeutic strategies.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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