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Involvement of testosterone signaling in the integrity of the neurovascular unit in the male: review of evidence, contradictions and hypothesis

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Short title: Testosterone and the neurovascular unit in males

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Abstract

Age-related central nervous system function decline and increased susceptibility of females, compared to males, with respect to prevalence of several neurodegenerative and neuropsychiatric diseases, are both based on the principle that hormonal factors could be involved. These cerebral disorders are characterized by an alteration of the blood–brain barrier (BBB) properties and chronic neuroinflammation, which lead to disease progression. Neuroinflammation, in turn, contributes to BBB dysfunction. The BBB and its environment, called the neurovascular unit (NVU), are crucial for cerebral homeostasis and neuronal function. Interestingly, sex steroids influence BBB properties and modulate neuroinflammatory responses. To date however, the majority of work reported has focused on the effects of estrogens on BBB function and neuroinflammation in female mammals. In contrast, the effects of testosterone signaling on the NVU in males are still poorly studied. The aim of this

review is to summarize and discuss literature, providing insights and contradictions to highlight hypothesis and the need for further investigations.

Introduction

The reproductive effects of sex hormones, namely estrogens and androgens, are well known. Furthermore, it is increasingly apparent that these hormones have major actions on non-reproductive systems such as the central nervous system, controlling homeostasis, development and some behaviors for instance ^{1,2}. These effects can be relevant throughout the lifespan of an organism, and are not limited to the reproductive period ¹⁻³. Additionally, they have a profound influence on both sexes and not restricted to one sex or the other ¹. One such example of the effects that sex steroids have on non-reproductive target tissues is their impact on cerebral blood vessels. Estrogens and androgens both regulate vascular tone, angiogenesis and endothelial cell survival, oxidative stress, and inflammatory responses in cerebral blood vessels ¹, but the actions of estrogens and androgens often oppose each other ⁴. Moreover, the expression of the respective receptors of estrogens and androgens suggest that cerebral blood vessels are directly targeted by sex steroids ⁵. Protective effects of other classes of sex steroids such as progestins, including progesterone, have also been documented ⁶.

Numerous therapeutic approaches aim to target the emerging effects of steroid deprivation and/or supplementation for amelioration of several CNS conditions ^{7,8}. Although many studies have investigated the therapeutic potential of estrogens and progesterone in blood–brain barrier (BBB) function in females ^{8,9}, much less attention has been devoted to clarify the impact of estrogens and androgens in males. The mechanisms underlying the effects of testosterone signaling on the neurovascular unit (NVU) in males are complex and still poorly studied. Currently, no review is available on this subject to our knowledge and data reveal evidence but also contradictions. Thus, the intention of our review is to synthesize the data of the literature to bring out hypotheses and highlight the need for further investigations.

We present a summary of the distribution of sex steroid receptors, mediating testosterone effects, in the male brain. Then, we review and discuss data concerning the effects of testosterone on cerebral micro-vessels, focusing on BBB permeability modulation. We also present an overview of testosterone involvement in neuroinflammation and oxidative stress responses and their relation with BBB alterations.

- **Blood-brain barrier**

In the central nervous system (CNS), cells require a stable microenvironment, which is guaranteed by a complex cellular and molecular system involving brain micro-vessels and their close environment referred to as the BBB^{10,11}. Indeed, the BBB is a highly regulated specific interface that maintains cerebral homeostasis to ensure neuronal activity. Indeed, the function of the BBB underlies the regulation of all vital functions of an organism. As such, BBB dysfunction can lead to many brain diseases and complicate recovery from injuries and insults^{11,12}. The walls of cerebral blood vessels are formed of endothelial cells (ECs) that are sealed by continuous intercellular molecular binding systems including adherens junctions (AJs) and tight junctions (TJs)¹³ that limit para-cellular diffusion¹⁴ (Figure 1). The major transmembrane components of TJs are the claudins, occludin and junction adhesion molecules (JAMs)¹⁵. They interact with the actin cytoskeleton via cytoplasmic TJs accessory proteins, including zonula occludens (ZO) proteins¹⁶ (Figure 1). In both mice and humans, claudin-3 and -5, ZO-1 and occludin have been described as the predominant proteins found at the TJs¹⁷. Claudins are crucial for the para-cellular sealing function, hence, they directly determine barrier function¹⁸. But the presence of claudin-3 in TJs of mouse BBB endothelial cells has been recently called into question: immunodetection of claudin-3 in mouse brain endothelial cells is thought to be an artifact due to antibody cross-reactivity with an undisclosed endothelial junction antigen still present in mice deficient in claudin-3.¹⁹ C57BL/6J mice lacking claudin-3 expression display an intact BBB and do not show any sign of BBB dysfunction¹⁹. Claudin-5 knockout mice, however, have a significant para-cellular ionic selectivity defect^{20,21}. A review published early in 2019 provides a comprehensive overview of the role of claudin-5 and its regulation in physiological and pathological conditions¹⁸. Likewise, disassociation of the ZO-1 junctional complex, which typically serves to stabilize TJs, leads to the breakdown of BBB integrity²². The AJs transmembrane components cadherin and nectin anchoring to cytoplasmic components such as catenin and afadin respectively, are also involved in the BBB integrity^{23,24} (Figure 1).

In addition to the intercellular binding systems that limit para-cellular diffusion, cerebral ECs also lack fenestration and exhibit extremely low rates of transcytosis, both of which greatly limit trans-cellular diffusion²⁵. Both features arise from several changes to ECs morphology and biochemistry that occur during embryonic development and allow the transition from a permeable to a continuous vessel²⁶. BBB is present throughout the CNS except in certain unique regions of the brain, the circumventricular organs. The circumventricular organs are characterized by fenestrated capillaries permeable to blood-borne molecules, and the interface ensuring the

cerebral homeostasis, in these cerebral areas, is constituted by specific cells called tanycytes. Those cells, lining cerebral ventricles, are sealed by tight junctions preventing the extravasation of molecules from fenestrated capillaries to the parenchyma via the cerebro-spinal fluid, forming thus the blood-CSF barrier (see for review ²⁷). Control of substance exchange between blood and cerebral parenchyma is also mediated by specific carrier systems present in EC abluminal or luminal plasma membranes (Figure 1). As such, the ability of macromolecules, fluids, and cells to cross endothelial barriers depends on charge, size, and binding characteristics ²⁸. Instead of passive diffusion, the supply of nutrients into the CNS occurs via active influx transporters such as glucose transporter 1: GLUT-1, whereas elimination of metabolites occurs via transporters of two major membrane transporter families, the ATP-binding cassette (ABC) family and the solute carrier transporter superfamily (SLC) ²⁹. Notably, efflux transporters (e.g., P-glycoprotein: P-gp, Breast Cancer Resistance Protein and the Organic Anion Transporter 3) are also responsible for the transport of several classes of pharmaceutical drugs ^{30,31}. Due to their restrictive nature, those efflux transporters can also be major obstacles in central distribution of many therapeutic agents for treating CNS diseases. Many lipid-soluble molecules, such as certain therapeutic drugs, are the substrates of efflux transporters, but some of them limit their absorption in order to protect the brain against potentially harmful exogenous substances ^{31,32}.

In addition to active protein transporters, endocytic vesicles serve as another major trans-cellular pathway accounting for BBB permeability in brain injury models ^{33,32} (Figure 1). Caveolae are non-clathrin-coated vesicles with plasma membranes enriched in caveolin (Cav) proteins, involved in receptor-independent transport of blood-borne molecules such as albumin ^{32,34}. To date, Cav-1, Cav-2 and Cav-3 isoforms have been identified in murine, bovine, and human cerebral endothelial and astroglial cells ³⁵.

The BBB represents a complex cellular system that consists not only of ECs, but also of pericytes embedded in basal lamina surrounding ECs, and astrocytic end-feet anchored to this basal lamina. ECs, pericytes, basal lamina, astrocytic end-feet and their neighboring microglial cells and neurons are collectively named the NVU (Figure 1); they govern BBB development, along with its maintenance and integrity ¹³.

Pericytes cover a large part of the abluminal surface (between 22% and 99% ¹⁰) of the endothelium and contribute to the stability of micro-vessels ³⁶. Their position and abundance within the cerebral capillaries suggest that these cells play a major role in BBB integrity. To date, it has been hypothesized that pericytes would deliver angiogenic factors that regulates permeability and vascular remodeling ^{37,38}, and that they would have contractile

capabilities to allow them to regulate blood flow through the capillaries³⁹. Moreover, pericytes play a major role in the differentiation of ECs during fetal life, even before the appearance of astrocytes⁴⁰. The recruitment of pericytes to endothelium and the interaction between the two are essential factors for the formation, maturation and maintenance of the BBB⁴⁰⁻⁴².

The basal lamina forms a continuous network of extracellular matrix (ECM) composed of type IV collagen, laminin, nidogen, heparan sulfate, elastin, and various proteoglycans and glycoproteins⁴³. The interaction between endothelial integrin, laminin, and other ECM proteins allows ECs to be anchored onto this basal lamina⁴⁴. In addition to its structural role, ECM degradation is associated with an increase in para-cellular permeability of the BBB⁴⁵.

Glial cells including microglial and astrocytes also represent an important element of the NVU. Microglial cells were described for the first time in 1919 by Del Rio-Hortega as a specific type of glial cell (see for review⁴⁶). When circulation begins to form at the CNS, at a time when the BBB is still permissive, some microglial cells precursors enter the CNS through the vascular network (see for the different hypotheses about the origin of microglia⁴⁷). Those microglial cells that enter the brain parenchyma during embryonic development, branch out then and remain quiescent⁴⁸. Microglial cells are activated during inflammatory reaction or immune challenge in the CNS and then differentiate into resident CNS macrophages^{45,49}. Upon immune challenge or following microglia activation, astrocytes also produce inflammatory cytokines^{50,51}. In addition, astrocytes are pivotal in the formation of tight junctions of the ECs, the promotion of the enzyme system γ -glutamyltranspeptidase involved in amino acid transport across the BBB, the differentiated distribution of transporters present in the luminal and abluminal plasma membrane of the ECs and ionic concentrations at the BBB⁵¹⁻⁵³.

A dysfunction of the BBB is associated with neurological damage and disease⁵⁴. Disruption of the BBB is also associated with peripheral diseases such as diabetes⁵⁵ and non-neural, inflammatory-related pathologies⁵⁶. Alteration of the cells of the NVU upon aging, inflammatory activation, oxidative stress, or neuropsychiatric and neurodegenerative diseases, leads to BBB dysfunction⁴⁴, which, in turn, contributes to the progression of neurological diseases^{17,44}.

- **Testosterone**

Testosterone was classically delineated as male-specific hormone according to its marked synthesis in the testes and its contribution to phenotypic and reproductive sex differences².

The biosynthesis of testosterone from cholesterol has been demonstrated in many tissues in addition to Leydig cells of the testes (in males). These include the brain (hippocampus) and liver and, to a lesser extent, the adrenal cortex zona reticularis and zona fasciculata in both males and females⁵⁷. Testosterone, which is a precursor of ovarian estrogens, is also present in females but at lower concentration (0.5–1 ng/ml in adult women vs 3–8 ng/ml in adult men)⁵⁸. Testosterone levels are sensitive to many internal and external factors, and mainly fluctuate with aging^{58,59}. Indeed, aging in men is accompanied by a progressive lowering of the serum testosterone level, in parallel with a decline in several CNS functions^{60–63}. This provides a logical reason to increase research to determine whether testosterone acts as a protective factor against several CNS disorders⁶⁴. In addition to age-related circulating testosterone changes, there are obvious gender differences in the prevalence of neuropsychiatric (e.g. depression, anxiety disorder, anorexia nervosa) and neurodegenerative (e.g. cognitive decline, autoimmune diseases, Alzheimer's (AD) and Parkinson's (PD) disease) diseases, with a higher incidence in females than in males^{8,64}. The reasons for gender differences, and a better understanding of how the state of being male limits these CNS disorders, are still unclear. It appears that sex bias is partly due to the protective effect of testosterone, or to the presence of the Y chromosome in males^{58,65}. A recent review has finely summarized the sex differences in cerebrovascular function, highlighting the evidences for sex steroid hormone-mediation in numerous cerebrovascular regulation and pathology⁶⁶.

Previously, testosterone research was almost entirely focused on reproductive issues. However, it is now known that testosterone is a regulator of fundamental physiological processes of non-reproductive tissues such as cardiac and skeletal muscle, or of development and function of the immune and nervous systems¹, including cerebrovascular tissue^{67–70}. Outside the brain, testosterone has a key effect on the cardiovascular system by the regulation of the vascular tone⁷¹. The greater incidence of clinical manifestations of heart disease in men and postmenopausal women compared with premenopausal women, translates a sex steroid hormones-induced gender difference for the vascular tone with a possible vascular protective effect of female sex hormones. It has been suggested that this gender difference in vascular tone is mediated at least in part by specific sex hormone receptors⁶⁸. Nevertheless, the role of testosterone in vascular contractility is debated. A number of studies have shown that testosterone facilitates vasoconstriction in rat (*in vivo* and *in vitro*)^{72,73} while other data have reported a vasodilator effect in rat and rabbit^{74,75}. Vascular mechanisms of testosterone have been thoroughly reviewed very recently⁷⁶. In brain, the regulation of cerebrovascular items is mediated through several mechanisms such as for instance, angiogenesis, vascular contractility, and control of the BBB permeability.

Distribution of sex steroid receptors, mediating testosterone effects, in the male central nervous system

Sex steroids, mediate their action via members of the nuclear receptor superfamily. These are expressed widely throughout the CNS by multiple cell types, but especially in brain areas involved in cognitive and reproductive behavior, and in neuroendocrine function². Testosterone exerts its effects on the organization and function of the CNS by either androgen receptors (ARs) or estrogen receptors (ERs) after undergoing neural aromatization to estradiol (E2; about 0.3%)^{1,77}. The reductase enzyme catalyzes the conversion of about 7% of testosterone to 5- α dihydrotestosterone (DHT), which is 2.5 to 10-fold more potent than the former^{78,79}. ERs- and ARs-mediated signaling pathways modulate several CNS activities by both genomic and non-genomic actions^{5,79}. To describe sex steroid receptor localization within the adult CNS, mRNA expression patterns of ARs and ERs were investigated in adult rats⁷⁹⁻⁸¹ (Figure 2). In males, nuclear ARs are expressed in the cortex and hippocampus (CA1 and CA2/CA3 regions), which control the regulation of cognitive behavior. However, ARs are also widely expressed in the olfactory bulb, the medial amygdala (MeA), the bed nucleus of stria terminalis (BNST), and the preoptic region (POA) of the hypothalamus involved in neuroendocrine functions and reproductive behavior⁷⁹ (Figure 2). It is also known now that nuclear ARs are present in neurons and glial cells as well as in cerebral blood vessels at the level of smooth muscle and the endothelium⁵.

Neural estrogens exert their effects through interaction with two nuclear ERs: estrogen receptor alpha (ER α) and beta (ER β). A comparative study provided evidence that nuclear forms of both ER α and ER β are expressed in the male rat CNS; some regions express exclusively ER α , for example at the level of the ventromedial hypothalamic nucleus (VMH), while others express only ER β , for example paraventricular nuclei. In contrast, other regions like BNST, medial and cortical amygdaloid nuclei, POA, periaqueductal gray and spinal trigeminal nuclei contain both forms of ER mRNA^{79,80}. The cerebral cortex and hippocampus also contain both ER mRNAs, giving a lower hybridization signal for ER α than for ER β . This suggests the involvement of ER β in cognition and memory^{2,79,80} (Figure 2). It is noteworthy that the highest level of ER α mRNA expression is in the hypothalamus and amygdala is related to regulation of sexual behavior and the hypothalamic-pituitary-gonadal (HPG) axis⁸².

In addition to their neural expression, specific receptors for gonadal steroids are expressed in the cerebrovascular tissue⁵. They can thereby control the integrity of the BBB by modulation of membrane transporters, intercellular molecular binding systems and neuroinflammatory responses^{67,69,83}, as we will discuss further below.

Additionally to their specific receptors, the metabolic enzymes for gonadal steroids have been found in cerebral blood vessels ⁵.

Testosterone -induced neuroprotection in the male central nervous system

As evoked above, gender differences in the prevalence of neurological disorders and diseases can be explained by neuroprotective effects of testosterone in males. Recently a pivotal role of androgenic signaling in modulation of neural activity within the CNS through myelination and re-myelination of neurons and synaptic plasticity has been documented ^{68,84}.

The decreased susceptibility of men, compared to women, to inflammatory autoimmune disorders such as Multiple Sclerosis (MS) is also described in some mouse models like the SJL/J mouse. This exhibits spontaneous myopathy, associated with an increased susceptibility to autoimmune diseases. Castrated SJL/J mice showed greater demyelination and inflammation within the spinal cord than sham-operated males ^{1,8,58,64}. Furthermore, recent data obtained in a mouse model of chronic demyelination ⁸⁵ and lysolecithin-induced demyelination ⁶⁴ have allowed the effects of androgenic pathway on myelin repair and re-myelination processes to be established. It is also well known that synaptic changes are involved in memory impairment occurring in the hippocampus of patients with AD ⁸⁶. A beneficial role of testosterone in improving cognitive performance in mice and rats by enhancing the hippocampal protein network which is critical for synaptic transmission and plasticity has been described ^{87,88}. Furthermore, the expression of ARs, not only in nuclei ⁸⁹ but also at extra-nuclear sites of hippocampal neurons ⁹⁰, has revealed the involvement of testosterone and DHT in neural activity and synaptic function. Gonadectomy also decreases density of spine synapses in the male rat hippocampus ⁹¹. Replacement therapy by added DHT or testosterone restores spinogenesis through AR signaling, which rapidly promotes kinase networks in neurons ⁹². In addition, despite the fact that there are no ARs in the dentate gyrus of the adult male rat hippocampus, it has been demonstrated that AR signaling induce neurogenesis via modulation of the survival of new neurons in this area of the brain ⁹³.

In many studies concerning psychiatric disorders, women are reported to have a higher incidence of depression than men ⁹⁰. Aging is also associated with an increased prevalence of depression in both sexes ⁹⁵. Depression can be associated with alterations in the proliferation and survival of hippocampal neurons: evidence indicates a role for androgenic signaling in the survival of new neurons in the hippocampus of young adult males ⁶⁴.

Chronic stress is also associated with reduced neurogenesis and expression of synaptic proteins in the CA1 and CA3 regions of the adult hippocampus ⁹⁶. The stress response is mediated through the production of glucocorticoids ⁹⁷ and it is well known that testosterone reduces glucocorticoids release ^{95,98}.

In men, the relatively high levels of circulating testosterone, compared to women, is correlated with a higher incidence of strokes ⁹⁹. It was shown that the high levels of testosterone and DHT, may contribute toward sensitivity to cerebral ischemia in young males ^{100,101}. Emerging experimental studies, however, have indicated that the decline of testosterone level upon aging in males can be associated with an increased risk of stroke ¹⁰². These data describe testosterone dose-dependent neuroprotective and neurodamaging effects in experimental models of cerebral ischemia. Finally, recent experiments *in vitro* have shown that gonadal hormones may be also involved in the suppression of neuronal apoptosis during ischemic disorders: testosterone and DHT suppress oxygen-glucose deprivation/re-oxygenation induced neuronal cell death in hippocampal slice cultures ¹⁰³.

In many brain diseases, disorders, trauma or stroke, neuronal damage is associated with an inflammatory response ¹⁰⁴ and a dysfunction of the BBB ¹⁰⁵. However, it has been shown in a model of MS a partial uncoupling of inflammation and neurodegenerative process¹⁰⁶. This study also suggests that a BBB dysfunction may persist regardless of inflammatory signals.

Recent data have suggested that the cross-talk between testosterone and glial cells, astrocytes or microglia, participates in the neuroprotective effect. This is achieved by attenuating neuroinflammation and oxidative damage during acute traumatic brain injuries such as stroke and hypoxia ^{58,107–109}. However, it has been hypothesized that the control of reactive gliosis such as an increase of astrocyte and microglia activation, a key event in neuroinflammation, is part of the testosterone neuroprotective mechanism ¹¹⁰. We will devote another part of this paper to a detailed discussion of testosterone effects upon neuroinflammation (see below).

Testosterone and cerebral blood vessels

Testosterone modulates vascular function through several mechanisms including, but not limited to, angiogenesis and BBB permeability.

- **Impact of testosterone on angiogenesis and vasculature formation**

The development of the BBB occurs in several steps, beginning with angiogenesis from preexisting vessels around embryonic day E10 in mice ¹¹¹ (Figure 3A). It has been demonstrated that testosterone modulates vasculature function and integrity via several mechanisms that involve vasculature formation, e.g. proliferation, migration,

and differentiation ¹¹². The patterns of circulating levels of testosterone in the male mouse from birth to adulthood are illustrated in figure 3B ¹¹³. Several researchers have focused their activities on testosterone impact on angiogenic mechanisms in cardiovascular regeneration and cerebral angiogenesis in adults ^{1,62,114}. However, the role of testosterone in CNS angiogenesis during fetal life has remained largely unexplored. In cultured human umbilical endothelial cells, testosterone induces endothelial cell migration through myosin activation and actin cytoskeleton remodeling ¹¹⁵. It also increases formation of endothelial cell tubes, by stimulating Smad1 phosphorylation. This represents one possible mechanism for testosterone-enhanced angiogenesis ¹¹⁶. An *in vitro* study using human dermal fibroblasts revealed that, DHT is a regulator of vascular endothelial growth factor (VEGF) secretion, the key mediator of angiogenesis in both CNS and non-CNS tissues ⁶². In addition, VEGF has a therapeutic potential for the induction of neuroprotection, neurogenesis, and cerebral angiogenesis after ischemic disorders such as stroke ¹¹⁷. Furthermore, histological and transcriptomic analyses of adult female robins and canaries treated with testosterone, reveal that this hormone induces angiogenesis in song control nuclei. This is associated with up-regulation of VEGF, VEGF receptors, and brain-derived neurotrophic factor (BDNF) ^{118–120}.

- **Impact of testosterone on cerebral endothelial cells and blood-brain barrier**

The impact of sex steroid hormones as therapeutic agents has received special attention with emphasis being placed on their neuroprotective effects. However, only a few studies have focused on the influence of sex steroid on the BBB and the NVU in both physiological and pathological conditions. Relatively little is known about testosterone compared to estrogens and progesterone due to: a) controversial results concerning its involvement on vascular endothelium function, b) debate as to therapeutic aspects - dose, duration, and health status, c) misuse or abuse for non-medical purposes, d) complicity of action due to interaction with multiple ERs. The maintenance of the BBB as a crucial interface, separating peripheral circulation and the CNS, requires perfect interaction between all components of the NVU. Additionally, altering critical properties of the BBB including, but not limited to, membrane transporters, vesicular pathways, basal lamina and TJ proteins of ECs could lead to a modification of microvascular integrity, hence, brain homeostasis. Testosterone modulates cerebral endothelial cells and blood-brain barrier function through the modulation of key elements of this complex cellular system.

- **Endothelial membrane transporters**

To date, only very limited data exist concerning testosterone effects on the regulation of membrane transporters at the BBB. OAT3, which mediates the efflux transport of uremic toxin and neurotransmitter metabolites such as dopamine metabolite¹²¹, is expressed at the BBB¹³. The expression of OAT3 in male rats is higher than in females¹²², and ARs regulate the expression and uptake rate of OAT3 in BBB cells (Figure 4)⁸³. It is noteworthy that the dopaminergic system participates not only in sexual behavior processes but also in regulation of cognitive behavior¹²³.

Sodium-dependent (SGLTs) and sodium independent (GLUTs) glucose transporters represent the two types of glucose transporter that regulate the uptake of glucose as a crucial energy source at the level of the BBB¹²⁴. The activity of these transporters is subject to modulation in a variety of neuropathological and neuropsychiatric disorders, including AD, PD¹²⁵, and depression¹²⁶. A study proposed that proinflammatory cytokine induce protein degradation of the number of glucose transporters, specifically, GLUT-1 and GLUT-3 in the BBB of AD patients (Figure 4)¹²⁷. It is notable that ovarian steroids are essential modulators of glucose transporters^{128,129}. To date, there has been no report concerning testosterone effects on these transporters' activities. Thus, the testosterone effects on membrane transporters at the BBB are not well understood, and this subject needs investigation. But it will be not surprising that testosterone could act as modulator of influx and/or efflux transporters partly through the modulation of inflammation mediators. Indeed, the impact of the Inflammatory mediators on BBB transporters has been described^{130,131}. For instance, the inflammatory mediators, such as tumor necrosis factor alpha (TNF- α), interleukin 1 beta (IL-1 β) and interferon gamma (IFN γ) can decrease the activity of P-gp at the level of the BBB (Figure 4)¹³². P-gp contributes to the efflux of brain-derived amyloid into blood. In addition, TNF- α reduces functional activity of BCRP (Figure 4)¹³³, which could alter the penetration of their substrates into the brain. Recently, evidence has shown that depletion of gonadal testosterone by castration of male mice induces up-regulation of IL-1 β and TNF- α in the preoptic area⁶⁷ (Figure 4), although the effects of these shifts upon membrane transporters were not evaluated.

○ **Trans-endothelial vesicular transport pathway**

As already mentioned caveolae are known as a major trans-cellular pathway in brain injury models. It was shown that the components of the caveolae plasma membranes, Cav proteins, play a critical role in signal transduction⁷⁷. In addition to brain endothelial cells, the Cav-1 protein has been described in vascular pericytes¹³⁴, while both Cav-1 and Cav-3 have been found *in vivo* and *in vitro* in reactive astrocytes, (Figure 4)^{134,135}.

It is important to address the controversy concerning the role of Cav-1 in regulating vascular permeability. An *in vivo* study in rats showed an enhancement of permeability of the BBB through up-regulation of the expression level of Cav-1³⁴. Nonetheless, data obtained from Cav-1 and Cav-2 knockout (KO) mice have shown a higher vascular permeability and lower lesion volume, respectively, after a stroke, compared to wild-type mice^{136,136}. This suggests a positive role for Cav-1 in the maintenance of the integrity of the BBB, in contrast to Cav-2. Cav-1 KO ischemic brains are also characterized by impaired angiogenesis and increased cell death¹³⁷. The role of Cav-1 in BBB integrity in neuropathology context and neural recovery after ischemic stroke was reviewed very recently, and the discrepancies in the literature pertaining to the role of Cav-1 were discussed¹³⁸.

Data based on rat immunohistochemistry analyses showed that an increase in Cav-1 expression at the cortical lesion site of cold-injury blood vessel model, is associated with loss of occludin and claudin-5 TJ proteins¹³⁹. However, it has been suggested that Cav-1 expression contributes to the recovery of TJ proteins and BBB function after injury. There are clear results indicating that TJ proteins are either bonded or localized in caveolae, and internalization, but not degradation, of occludin and claudin-5 through Cav-1 is responsible for alterations in brain endothelial barrier permeability during inflammation¹⁴⁰. Recycling of internalized TJ proteins is crucial for recovery of the brain endothelial barrier integrity¹⁴⁰ (Figure 4).

Despite confusion in the current literature concerning the roles of Cav-1 in modulation of the BBB integrity, thus far studies have provided evidence that Cav-1 could prevent oxidative and inflammation injury, and extracellular degradation of TJ proteins and matrices^{140,141}. The increase in Cav-1 upon brain injury may reflect its beneficial involvement in the recovery of BBB integrity in damaged CNS but would not explain a detrimental role. We can also suppose that the effects of Cav-1 may be isoform, tissue, state of health and dose dependent. Further, Cav-1 may have different functions in different neurological dysfunctions. The exact mechanisms and explanations for these controversial results remain unclear and this is an issue that needs further investigation.

There is evidence showing that non-genomic ARs can interact with numerous signaling molecules at the plasma membrane. Thus, ARs interacting with caveolae, trigger numerous kinase-signaling pathways involving in particular the Src kinase, extracellular signal-regulated protein kinase, the phosphoinositide 3-kinase and the protein kinase B⁷⁷. Cav-1 protein is known as ARs co-activator^{142,77}, allowing this nuclear receptor to efficiently mediate gene expression. Thus, it cannot be excluded that AR non-genomic activity indirectly influences activity of either genomic ARs or other nuclear receptors. Furthermore, a recent study showed a positive correlation

between the level of Cav-1 and ARs transactivation and sensitivity of ARs to ligand-dependent activation, confirming the role of Cav-1 as a convergent point for ARs cross-talk with other cellular signal transduction pathways (Figure 4)¹⁴³. Thus, given the importance of Cav-1 in modulation of the BBB integrity and its links with ARs, study of the molecular mechanisms underlying the interaction between the Cav-1 protein and the androgen receptor requires further investigation.

- **Basal lamina and the immediate surroundings of endothelial cells**

The basal lamina as an acellular membrane surrounding ECs has a pivotal role in BBB integrity¹⁴⁴. Loss of the basal lamina stability triggered by metalloproteases or collagenases is frequently observed in pathological manifestations involving the BBB breakdown¹⁴⁵.

Nitric oxide (NO) is a diffusible gas, which at a basal concentration is dose-dependently capable of regulating numerous physiological functions (neuronal communication, vascular tone regulation etc.), and at higher concentrations can cause disease (cell death) through multiple mechanistic pathways¹⁴⁶. The basal concentration of NO derives from endothelial nitric oxide synthase (eNOS) enzyme activity, whereas deleterious effects are due to the activity of neuronal and inducible NOS isoforms (nNOS and iNOS respectively)¹⁴⁶.

NO mediates its physiological effects at least in part by the activation of matrix metalloproteinases (MMPs) during matrix reorganization¹⁴⁶. MMPs 2, 3, and 9 are the main forms found in the brain, and are the prime proteolytic enzymes particularly involved in the degradation of the ECM¹⁴⁷ and in neuron damage¹⁴⁸⁻¹⁵⁰. Activated MMPs also lead to BBB leakage by catalyzing hydrolysis of the TJ proteins, thereby inducing their degradation¹⁵⁰⁻¹⁵² (Figure 4). In addition, both MMP2 and MMP9 contain Cav-binding motifs and there is direct evidence that Cav-1 can down-regulate MMP activities and decrease BBB permeability in brain microvascular ECs during cerebral ischemia-reperfusion injury¹⁵³. Moreover, Cav-1 protein regulates the activation of NOS by binding to all NOS isoforms that contain the Cav-binding motif, thereby inhibiting NO production (Figure 4)¹⁵³. In a recent investigation, the Cav1/NO/MMP pathway has been suggested as a therapeutic target to ensure protection against cerebral ischemia-reperfusion injury (Figure 4)¹⁴¹. Given the importance of the links between ARs and Cav-1, as mentioned previously, it could be hypothesized that AR would be another important partner of the Cav1/NO/MMP pathway, ensuring BBB integrity.

Pericytes are located around the cerebral capillaries, and they share the basal lamina with ECs. Although a decrease in pericyte numbers and ultrastructural changes in aging and age-related brain diseases such AD, in both human and preclinical models ¹⁵⁴, there is no data concerning the effects of sex steroid hormones on pericytes. Further investigations in this regard are clearly required.

- **Inter-endothelial tight junctions**

Cerebral ECs possess unique phenotypic properties due to the presence of continuous TJs, the lack of fenestration, minimal pinocytotic but more peptidase and mitochondrial activity ^{44,111}. Numerous biochemical components or pathophysiological states, can directly or indirectly disrupt the NVU at both the structural and functional level. Such disruption leads to an increase in leakage across the BBB ⁶⁹.

Abnormal cerebrovascular TJs have been detected in a wide-range of diseases including ischemic stroke traumatic brain injury, AD and MS ⁴⁴, as well as in physiological aging ¹³.

It is now well known that ovarian hormones influence the BBB integrity, with respect to limiting para-cellular permeability via the preservation of TJ proteins ^{69,155}. However, the contribution of testosterone to the BBB TJs response is poorly defined.

The first conclusive evidence that under physiological conditions, testosterone, impact TJ protein expression in males was obtained in our 2017 study. This showed that the castration of adult male mice significantly decreased the expression of TJ proteins (claudin-5 and ZO-1 but not occludin) in the hypothalamic POA ⁶⁷, leading to changes in morphological arrangements and /or delocalization and disorganization of brain endothelial junction proteins. Additional results based on different molecular weight tracers, as a marker of BBB integrity, suggested that chronic depletion of testosterone induces defects in the BBB permeability in this region. The supplementation with testosterone after castration restores the BBB impermeability and TJs integrity ⁶⁷. In connection with this point, the specific roles of the neural ARs and their signaling pathways are being studied. These results can be compared to those obtained in mice with a selective deletion of ARs in Sertoli cells, which reveal that ARs in Sertoli cells are pivotal for timely and complete blood-testis barrier formation. The absence of functional ARs represents a potential cause for delayed and defective (leaky and incomplete) barrier formation ¹⁵⁶. Thus far, these results suggest that androgenic signaling supports the BBB integrity. However, this study does not shed light on whether, genomic or non-genomic steroid pathway, mediates androgen effects.

Testosterone and neuroinflammation: relationships with blood-brain barrier dysfunction

Inflammation and innate immunity are believed to contribute to the occurrence and severity of pathogenesis and neurodegenerative diseases. Furthermore, as already mentioned, microglial activation that is involved in neuroinflammation represents one of the most frequent clinical characteristics of these disorders. In addition, reports have already pointed out rapid and progressive alterations of the brain endothelium during such diseases. Interestingly, testosterone does not only regulate the development and function of the immune system, but also modulate neuroinflammatory responses. These should be neither underestimated nor neglected. In the context of neuroinflammation, further research has focused on the apparent roles of resident cerebral cells, including astrocytes and microglia.

This activation of microglia is directly associated with a dysfunction of the BBB^{157,158}. Microglia express a cytoplasmic marker, the Iba-1 protein (ionized calcium binding adapter molecule 1), which is upregulated during microglial activation^{159,160}. The degree of activation of microglia appears to be correlated with the type and severity of the brain disorder¹⁶¹. Thus, activation is part of their primary action as mediators of responses to pathogens and injury. They have either protective (housekeeping functions) or destructive (buffering action) impacts on the CNS, depending on their state and the duration of activation¹⁶². Microglia known as the source of pro-inflammatory cytokines (e.g. TNF- α , IL-1 β , IL-2, IL-4, IL-6), prostaglandins, chemokines, enzymes associated with inflammation (e.g. iNOS and COX-2) as well as inflammatory cytokines production¹⁶³. By releasing cytokines such as TNF- α activated microglia induces neurotoxic reactive astrocytes (termed "A1") which might have harmful functions¹⁶⁴. Numerous studies indicate that following CNS injury, reactive astrocytes upregulate both vimentin and glial fibrillary acidic protein (GFAP)^{165,166}. Astrocyte-inducible NOS has been described to play a major role in human neuroinflammation¹⁶⁷⁻¹⁶⁹.

Gliosis corresponds to the reactive change of glial cells (microglia and astrocytes) in response to damage^{170,171}. This is conjugated with the modulation of local chemokines and cytokines, which govern inflammation (figure 4). Chronic gliosis may heighten the neurodegenerative process. Thus, neuroglia have been identified as a therapeutic target to halt inflammation in several neurodegenerative diseases¹⁷².

The action of testosterone on reactive gliosis is not yet well-documented. However, available evidence indicates that administration of testosterone, but not DHT, down-regulated reactive gliosis after brain injury in male rats^{110,173,174}. Recently, it has been shown that reactive astrocytes induce BBB disruption at least in part via the production of VEGF-A and thymidine phosphorylase (TYMP) in the case of inflammatory brain lesions¹⁷⁵.

Furthermore, we have shown that castration of male mice is associated with activation of neuroglial cells. This occurs via up-regulation of inflammatory proteins such as COX-2 and iNOS as well as pro-inflammatory cytokines such as TNF- α . Interestingly, supplementation of testosterone after castration suppresses these inflammatory features⁶⁷. Data observed in a variety of cerebrovascular diseases and neurodegenerative disorders showed that the function of the BBB is compromised once a state of neuroglia activation is attained. This is accompanied by an increase in the expression and release of several pro-inflammatory molecules such as TNF- α and IL-1 β ¹⁷⁶. In addition, it has been proposed that in turn, BBB dysfunction contributes to the progression of neurodegenerative diseases¹⁴. However, due to lack of a key pathway, the link between neuroinflammation and disruption of the BBB is still poorly understood, and it remains difficult to determine whether BBB breakdown is a cause or a consequence of the inflammatory status.

Testosterone and mitochondria-derived oxidative stress: relationships with blood-brain barrier dysfunction

The sensitivity of the BBB to oxidative stress is due to a high mitochondrial content (8 to 11% of the endothelial cytoplasmic volume) in cerebral endothelial cells, which is pivotal to maintain active transport¹⁷⁷. Nevertheless, information concerning testosterone impact upon mitochondrial reactive oxygen species (ROS) generation in the BBB is still scarce.

The ROS and reactive nitrogen species (RNS) free radicals are derived from both endogenous and exogenous sources. They are well-recognized for dual roles, acting as both deleterious and beneficial species. The mitochondrial respiratory chain is an important endogenous source and target of ROS¹⁷⁸ (figure 4). It is noteworthy that the intrinsic apoptotic pathway is mediated by mitochondria through ROS production. Increased ROS production induces oxidative stress, which has been reported to be involved in several pathological conditions such as cardiovascular disease. Furthermore, ROS activates inflammation factors¹⁷⁹. Interestingly, the mitochondria impairment disrupts EC TJs and increases BBB permeability, which is accompanied by deterioration as an outcome of neurodegenerative disorders (e.g. stroke, PD and AD)¹⁸⁰. In addition, oxidative stress induces inflammation, which can further increase oxidative stress activity (Figure 4)¹⁸¹. Low testosterone levels^{182,183}, aging¹⁸⁴, and testosterone-deprivation¹⁸⁵ are risk factors for oxidative stress. It was shown that there is an inverse association between testosterone concentrations and oxidative stress markers in adult men¹⁸⁶. In the CNS, there is evidence to indicate that testosterone improves mitochondrial membrane potential, and reduces nuclear fragmentation and ROS generation in a human astrocyte cell model, acting via positive regulation of neuroglobin. The latter represents a key factor involved in cellular oxygen homeostasis¹⁸⁷. To date, the

involvement of testosterone in oxidative stress at the level of the NVU is still not well-established, and needs further investigation. Effects of testosterone upon the oxidative status are still controversial, ranging from ROS generation to scavenging or chain-breaking of ROS and RNS ¹⁸⁸⁻¹⁹¹. Numerous studies have indicated that the effect of testosterone in this regard is tissue, sex and dose dependent ¹⁹². In prostate cancer cell lines, androgen signaling regulates the cytoplasmic dynamin-related protein, a key protein of the mitochondrial fragmentation machinery, that leads to cell death but not to cell division ¹⁹³. Moreover it was shown that, in these cell lines, testosterone causes the ROS and H₂O₂-induced apoptosis ¹⁹⁴. Like cerebral endothelial cells, gastric parietal cells contain many mitochondria. Data based on gonadectomized male rats, following testosterone supplementation, suggested that testosterone could mediate mitochondria-associated apoptotic signaling in the gastric mucosa by reducing ROS generation and by impairing apoptosis processes ¹⁹⁵. However it has been reported that androgens induce ROS generation through AR signaling in cultured vascular smooth muscle ¹⁹⁶ and renal interlobar arteries ¹⁹⁷.

Conclusion

The aim of this review was to present an overview of the data of the literature concerning the effects of testosterone on the NVU in males. It mainly emerges from these studies that i) testosterone promotes cerebral angiogenesis and vasculature formation, ii) testosterone supports the integrity of BBB TJs and, iii) regulates some endothelial membrane transporters although very little data is available yet. The effects of testosterone are mediated by either androgen or estrogen receptors and the mechanisms by which it regulates these features are usually not described. Nevertheless, ARs and their interaction with the Cav-1 protein would be involved in the BBB integrity by participation in Cav-1/NO/MMPs pathway.

A dysfunction of the BBB is often associated with an inflammatory status inducing a neurodegenerative process. But the link between neuroinflammation and disruption of the BBB is still poorly understood. Data suggest that testosterone could prevent from gliosis reaction and up-regulation of inflammatory proteins, and could participate in reducing ROS generation and limiting oxidative stress.

To conclude, data from literature show an undeniable role of testosterone on brain capillaries guaranteeing the cerebral homeostasis in males but they reflect complex actions of hormones which appear to be brain region,

state of health and/or dose-dependent. The cellular and molecular mechanisms underlying the maintenance of the BBB integrity and the associated signaling pathways remain to be analyzed in greater details.

Understanding the role of testosterone and testosterone signaling in the functions of cerebral blood vessels and the BBB could lead to the exploration of the therapeutic potential of testosterone in several CNS disorders. Indeed, this could answer partly, the enigma of gender differences and in age-related emergence and progression of some CNS pathologies or dysfunctions.

It is also important to continue to explore this area of research, as this will lead to a better understanding of the action of environmental contaminants such as endocrine disruptors that interfere with normal hormonal balance and/or pathway and thus exert potentially deleterious effects on health. Considering the abundance of environmental contaminants that have anti-androgenic and/or anti-estrogenic properties, the consideration should be given to the effects of these components on brain micro-vessels.

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Figure Legends

Figure 1. The major cellular elements of blood-brain barrier localized at central nervous system micro-vessels are comprised of a continuous layer of endothelial cells, basement membrane cells, pericytes, microglial cells, neurons and astrocyte end-feet. BCRP, breast cancer resistance protein; GLUT-1, glucose transporter 1; JAMs,

junction adhesion molecules; OAT-3, organic anion transporter 3; P-gp, P-glycoprotein; ZOs, zonula occludens proteins.

Figure 2: Overview of a whole male brain with a close-up view of the position of hypothalamic nuclei pointing out the relative location and density of androgen receptor (blue circle) and estrogen receptor (ER α , green square; ER β , pink diamond) mRNA distribution using in situ hybridization analysis in male rat ^{79–81}. AC, anterior commissure; AHN, anterior hypothalamic nucleus; AMe, medial amygdala; ARC, arcuate nucleus; BNST, bed nucleus of stria terminalis; DHN, dorsomedial hypothalamic nucleus; Hip, hippocampus; Hyp, hypothalamus; L, lateral; LN, lateral nucleus; LPN, lateral preoptic nucleus; M, medial; MPN, median preoptic nucleus; OB, olfactory bulb; OC, optic chiasma; PN, periventricular nucleus; POA, preoptic area; PPN, periventricular preoptic nucleus; PVN, paraventricular hypothalamic nucleus; SCN, suprachiasmatic nucleus; SON, supraoptic nucleus; TH, thalamus; TMN, tuberomammillary nucleus; VMN, ventromedial nucleus.

Figure 3: A: Developmental timeline of the blood-brain barrier set up in the murine central nervous system ¹¹¹. B: pattern of circulating level of testosterone in the male mouse from birth to adulthood ¹¹³. E: embryonic days.

Figure 4: State of the art schematic representation of testosterone signaling-induced cellular and molecular interactions in the blood-brain barrier system, highlighting in particular the Cav-1/NO/MMPs pathway involvement. Testosterone controls the integrity of the BBB by preserving the intercellular molecular binding proteins degradation, by regulating endothelial transporters expression or activity, and by decreasing inflammatory response. Cav-1 protein is known as an ARs co-activator. Cav-1 protein is expressed by brain endothelial cells and pericytes, while reactive astrocytes express both Cav-1 and Cav-3. Cav-1 is responsible for 1) internalization and recovery of TJ proteins during a brain insult 2) inhibition of NO production and consequently inhibition of MMPs activity, thereby preventing TJ protein hydroxylation, ECM degradation and neuronal loss.

Briefly, the CNS response to insult and injury is manifested by gliosis. The inflammatory responses are directly associated with a neuronal loss, degradation of TJ proteins, decrease of the number of GLUT-1 and decrease of the activity of P-gp and BCRP transporters. Testosterone, in turn, decreases inflammatory mediators. In addition, ARs regulate positively the expression and uptake rate of OAT3. Oxidative stress caused by an over-production

of ROS, activates inflammation factors. The cross-talk between oxidative stress and neuro inflammation causes BBB leakage. The involvement of testosterone in oxidative stress is not well-known.

ARs, androgen receptors; BBB, blood-brain barrier; BCRP, breast cancer resistance protein; Cav, caveolin; ECM, extracellular matrix; GLUT-1, glucose transporter 1; IFN γ , interferon gamma; IL-1 β , interleukin 1 beta; MMPs, matrix metalloproteinases; NO, nitric oxide; NOS, nitric oxide synthase; OAT-3, organic anion transporter 3; P-gp, P-glycoprotein; ROS, reactive oxygen species; TNF- α , tumor necrosis factor alpha; ZOs, zonula occludens proteins.

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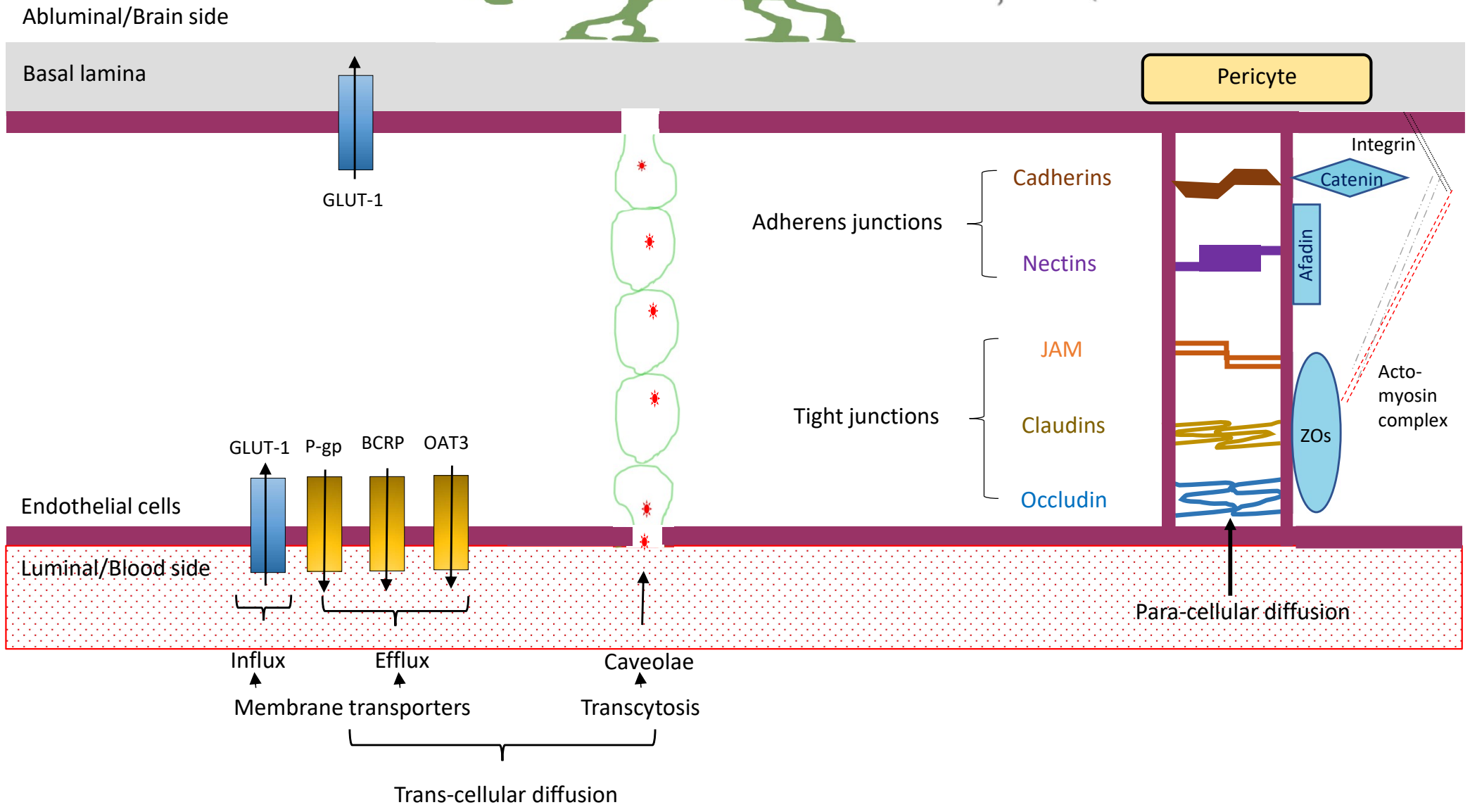
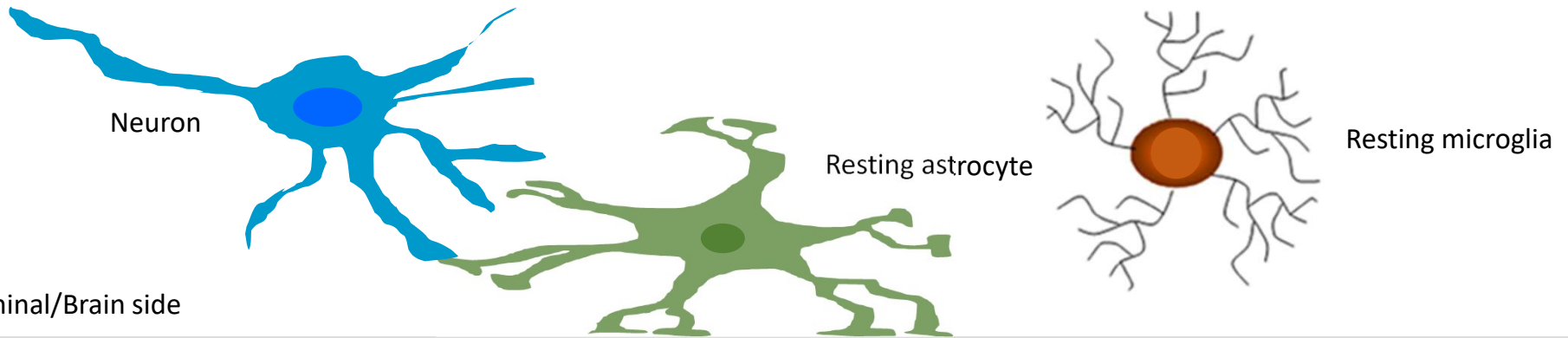
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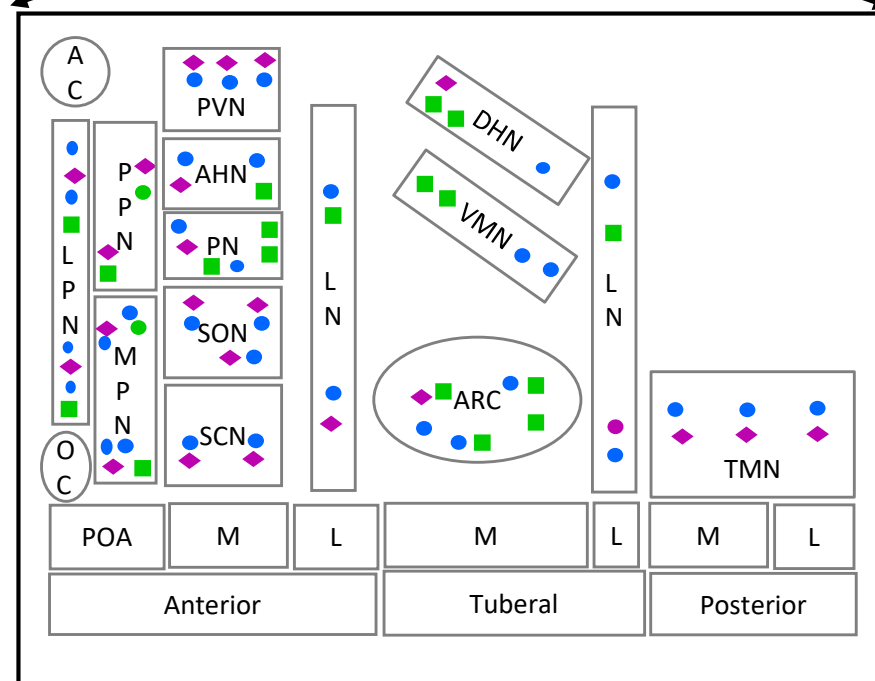
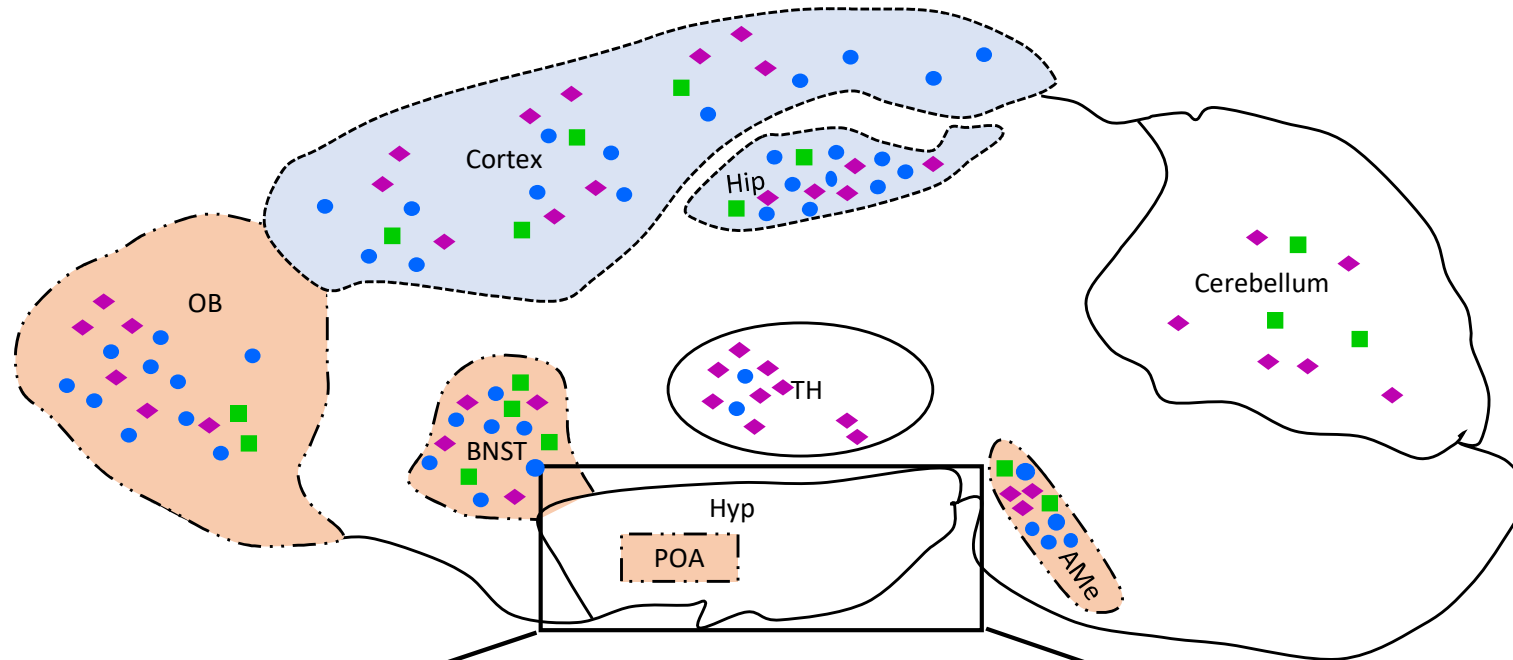
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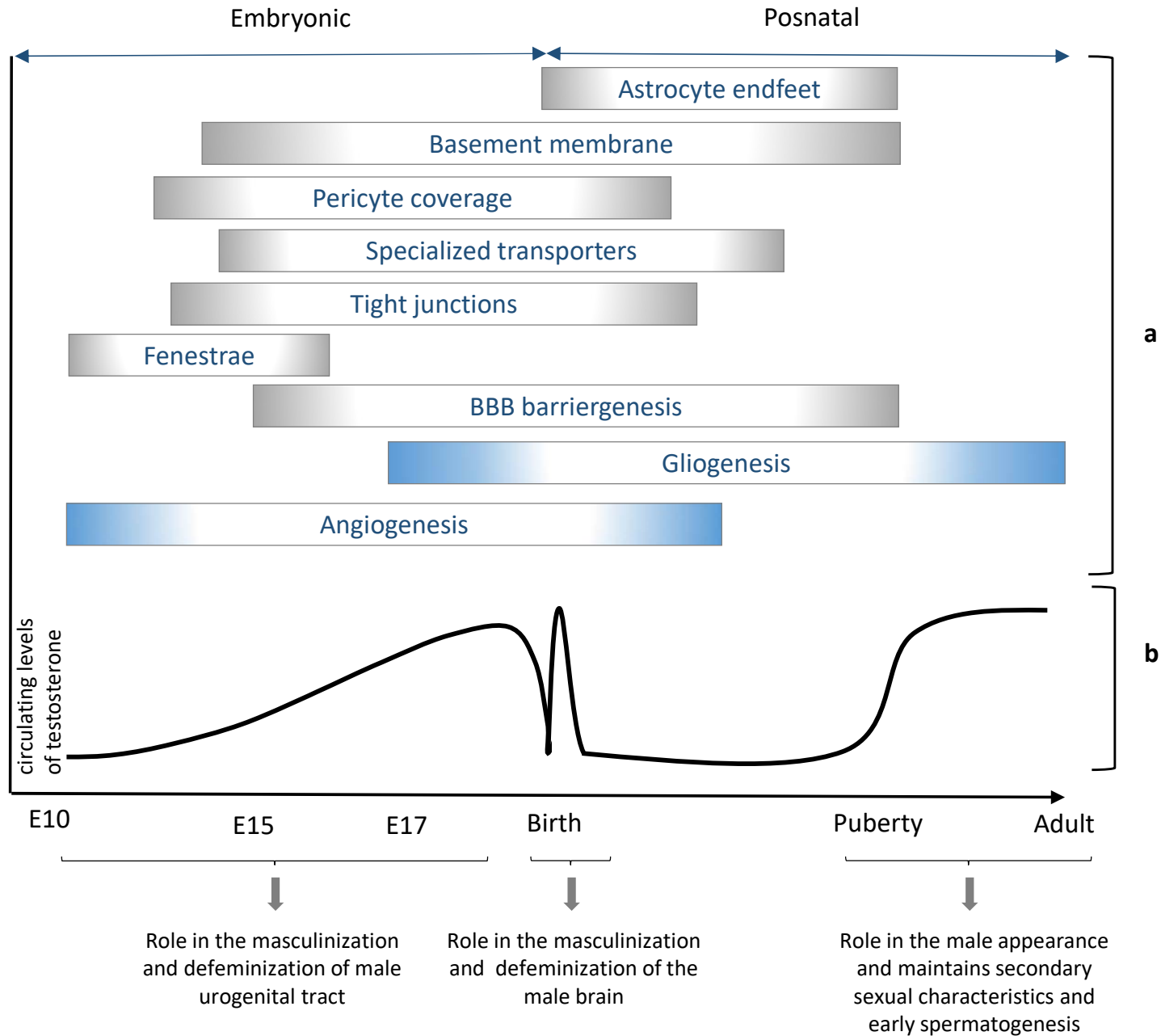
Brain regions involved in memory and cognition

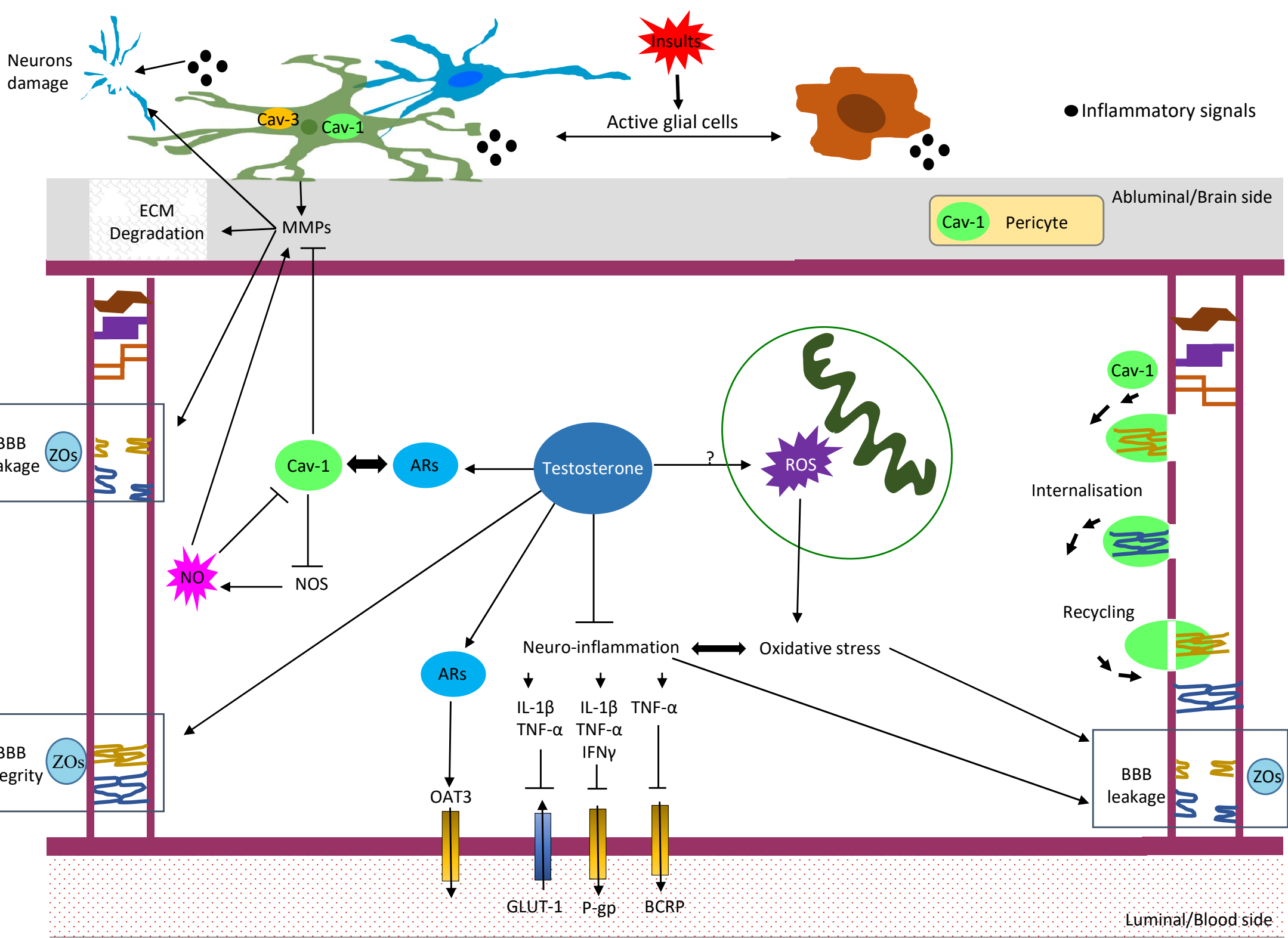
Brain regions underlying sexual behavior

AR ●

ER α ■

ER β ◆





Neurons damage

Insults

Active glial cells

● Inflammatory signals

ECM Degradation

MMPs

Abluminal/Brain side

Cav-1 Pericyte

BBB leakage ZOs

BBB integrity ZOs

BBB leakage ZOs

Luminal/Blood side

Testosterone

Cav-1

ARs

ROS

NO

NOS

Neuro-inflammation

Oxidative stress

Internalisation

Recycling

ARs

IL-1 β

TNF- α

IL-1 β

TNF- α

TNF- α

IFN γ

OAT3

GLUT-1

P-gp

BCRP