



Effects of phthalates, environmental endocrine disrupting chemicals, on the neurovascular unit in adult male mouse

Delnia Ahmadpour

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Delnia Ahmadpour. Effects of phthalates, environmental endocrine disrupting chemicals, on the neurovascular unit in adult male mouse. *Neurons and Cognition [q-bio.NC]*. Sorbonne Université, 2021. English. NNT : 2021SORUS155 . tel-03600952

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THESE DE DOCTORAT**DE SORBONNE UNIVERSITE**

Dans le but d'obtenir le titre de

DOCTEUR DE SORBONNE UNIVERSITE

Spécialité Neurosciences

Ecole doctorale CERVEAU, COGNITION COMPORTEMENT

Présentée par Delnia AHMADPOUR

**Effects of phthalates, environmental endocrine disrupting chemicals,
on the neurovascular unit in adult male mouse**

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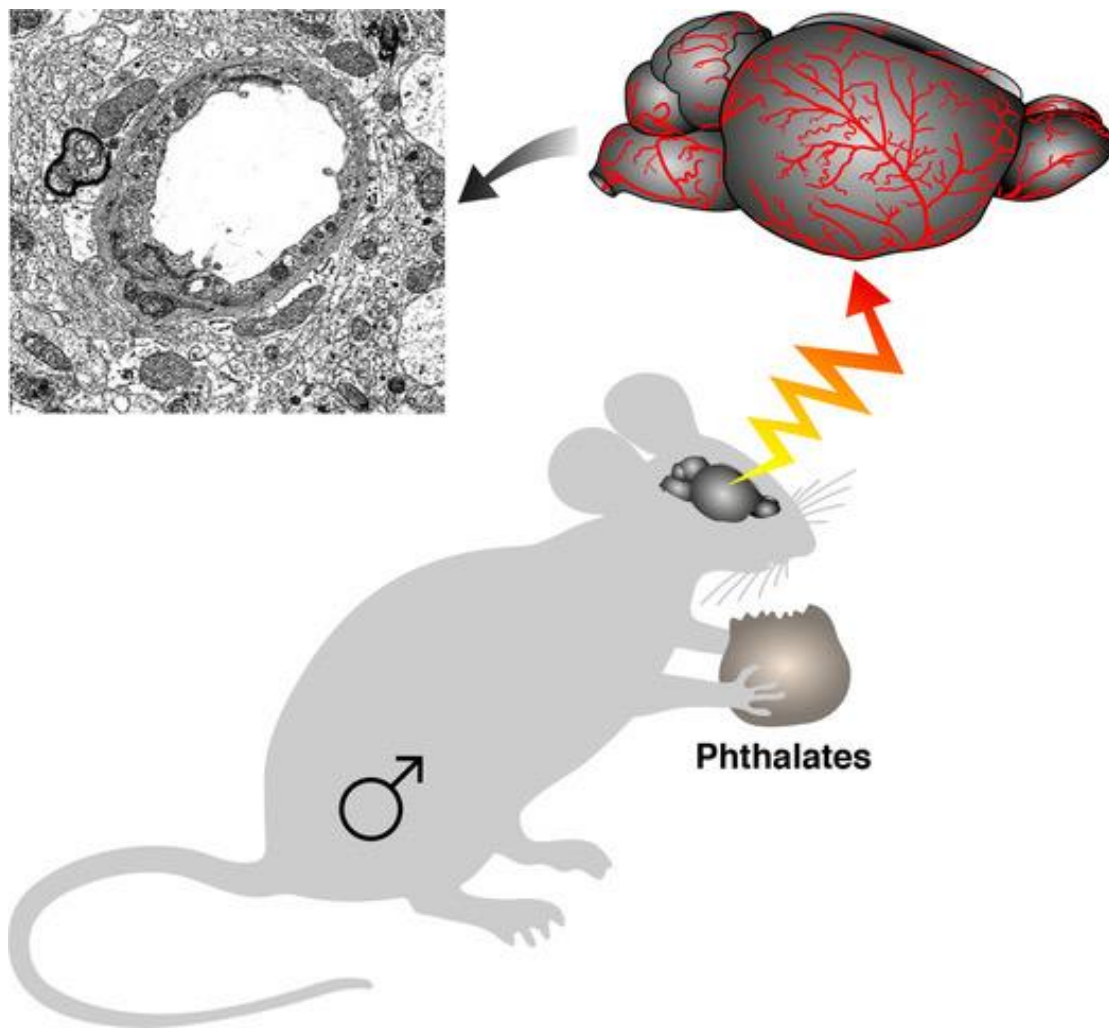


Illustration by Sophie Gournet, IBPS, CNRS UMR7622

Acknowledgements

“What matters in life is not what happens, but what you remember and how you remember it”, said Garcia Marquez. While many memories of the last three years have faded surprisingly quickly, others are most precious. Although the following dissertation may seem like an individual work, I can fully guarantee that I am not strong enough to carry all these heavy stuffs on my own. I have received assistance and support from several people whom I want to thank because they have helped me reach this point.

First and foremost, I would like to thank all the jury members for agreeing to evaluate my thesis work.

Dr. Benedicte Dehouck and Pr. Jean-baptiste Fini; I wish to take this opportunity to thank you once again for your previous participation in my dissertation committee in 2020. I would like to thank you again for accepting to review this manuscript.

Pr. Anne Duittoz and Dr. Pascal Vaudin; I would like to express my deepest and my utmost gratitude, not only for agreeing to be part of my jury, but also for being such great professors. I will never forget how friendly you were and I am forever grateful for your guidance and support throughout my master.

Pr. Rachel Sherrard; A special thank you for agreeing to join the jury members and for tacking the time to read this manuscript.

My supervisor, Dr. Valerie Grange-Messent; I would like to express my extreme sincere gratitude. Valerie, this dissertation would not have been possible without your continuous support and guidance. I deeply appreciate all your contributions of time, scientific advice, presence and availability, ideas and consistent support during the running of this project. It was a great privilege to work under your supervision, a noble and honorable human being with immense knowledge and skills. You continuously provided encouragement and were always willing and enthusiastic to assist me in any way you could. Thank you for letting me follow you around at conferences, for believing in me enough, for giving me the freedom to express my ideas

and allowing me to make mistakes, thus learn. Your confidence means more to me than I can express. I will never thank you enough for what you have been and still are for me and I am totally aware of how bloody lucky I am! Thank you for this wonderful thesis (of course it is) that we accomplished together and I hope that our collaboration will last for many more years.

My sincere thanks also go to Dr. Sakina Mahouty-Kodja who provided me with the opportunity to be part of this incredible research team. Sakina, I have kept your email in which you gave me a positive response to realize my thesis in your laboratory. I thank you deeply for your continuous help, support and scientific advices. I am really grateful for the time you devoted for the rigorous editing of my papers.

I would like to express my deepest and sincere thanks to Dr H  l  ne Pouzet, for her encouragement and thoughtful and constructive remarks during the lab meeting. Helene I deeply appreciate the openness, kindness and generosity that you have shown me when I needed it. I will never forget your presence during the quarantine. Thank you again and again and again.

I am grateful to our engineer Caroline Parmentier; Caroline, I would like to express my gratitude for your technical advices, countless gifts and your generosity. God knows that I am going to miss your "g  teaux" and your "p  tes de fruits". Thanks again Caroline for sewing the pretty masks for all of us during the mask shortage. You are the best. You managed it better than the president Macron administration.

Amazing Lydie Naul  , I've heard so much about you and now I am realizing why! It is easy, because you are great! I am very glad to see you here and wishing you good luck in your research work.

Nolwenn and Suzanne (Adampour and Ducroqpour); Thank you for putting up with the millions of questions I have asked you and for listening to me talk about my problems. Thanks for taking care of my mice in my absence. I will never forget the times of the "boulettes", the times spent at the "B7", the times of the "pause caf  " and "Bi  re". My good, great genius Nolwenn, there have been so many memories and I ca'tn wait to do more. I am the funniest friend in the world and the greatest singer in the world, according to you! My dearest generous grounded

Suzanne, thank you for all the laughs during these three years, I am blessed to have spent almost three years with you, in the same office, side by side. Hey Suzanne (repose toi! clin d'oeil, clin d'oeil). Girls, words cannot describe the joy and happiness I felt seeing your results or publications. You rock and your research is as fabulous as you are. I almost forgot, I will deliver your Kurdish green card to you as soon as possible. You will quickly realize that it is not a gift that I gave you. Anyway, be faithful and “bonne intégration”.

“Ma petite” Marie Amelie, “mon petit” Elliott and mia bella Francesca, thank you very much for your support and cooperation. You guys made the laboratory’s life going easier with your energy and your good mood. I will leave you the heritage of Western blot; Enjoy!

How sad is the hallway of A3 without your presence “Des bons vivants du A3”; Melanie Marias, Marie Charlotte Allichon, Guillaume Ubiema, Virgile Delaunay, Theo Andriot. I fall short of words to thank you. You constantly made time to showing me all of the techniques of your laboratory and answer any and all questions I had, even the dumb ones. Thanks for giving me beautiful memories that I am going to cherish for a lifetime. I could not ask for more. Your unconditional love means the world to me because getting through my dissertation required more than academic support. My heart is filled with love and respect for every single of you and I am definitely going to miss you.

Uz, we are lucky to have such a kind and generous person in the hallway of A3. You put a really big smile on my face every time I run into you. Thank you, “Monsieur”.

Philippe Glaize; It’s unbelievable and slightly unfair how great you are. I can’t thank you enough for everything you have done over the last five years to help me reach this point. Thanks for your support and encouragement that were constantly available, not only during my academic life but also during my personal life. Also, thank you for patiently listening to me talking about my research even though you had no idea what I was talking about. Thanks for making sure that I had enough “du bon rosé de Côtes de Provence” “du bon vin pétillant de la Loire” and “du bon fromage fermier de chèvre de Touraine” in my fridge. Thanks for pointing out my mistake in French speaking with your famous phrase (N’iiiiimporte quoi, ben alors là, franchement, mais ce

n'est pas vrai). Philippe, sacré Philippe, si tu savais à quel point je t'apprécie. Si continue je pleure!
Allons, passons!

Isabelle sweetheart, thank you for your kind gestures, in Perpignan or in Paris, I will never forget it throughout my life.

My beloved family, my father Jalal giyan, my mother Khatoon giyan, my brothers Saman and Simko giyan, mes “bawan” I am exhausted and the only thing that could relieve me is to take refuge into your arms. You have no idea how it was unfairly difficult. But I did it, as I promised, as you wanted.

My adorable friends, Mahdi giyan, Shivan giyan, Msto giyan, Parastou giyan, Homa giyan, mes “haoreyan” I am glad that the love in our friendship is not dependent on mood swings and bad hair days. Thanks for being best friend ever.

My lovely Saeid giyan, “khoshavist” thank you for fighting all of the life’s battles by my side. As said by Jacques Brel “ on se dit souvent qu’on aura une maison, avec des tas de fenêtres avec presque pas de murs, et qu’on vivra dedans, et qu’il fera bon y être, et que si c’est pas sûr c’est quand même peut-être... »

Nazand Bagikhani giyan; having you is a blessing. The more time I spend with you I realize what a wonderful and caring person you are. Thanks for lending your ears and listening to my rants and thanks for your support.

Serhet and Emir giyan, mes “hevalno” thanks for helping me to move out four times in two years, thanks for taking care of everything, fixing my bike after the crash, furniture installation and of course, insisting that I may know things about science, but I’m definitely clueless when it comes to do DIY stuffs.

Marie-Agnès, Thank you for all your support over the past six months. I am deeply touched by your warmth and hospitality and I am thankful for your unconditional generosity.

I cannot express my gratitude enough to Kurdish Institute of Paris and Campus France for the five years' financial support that was incredibly helpful. This scholarship significantly reduced my financial burden. I hope that I will be able to follow your footsteps and give back to others in all the ways possible.

I want to express my deep thank to all of you and anyone I may have forgotten.

Abstract

We have previously shown that adult male mice exposure to low doses of ubiquitous endocrine disruptor of phthalate family, di(2-ethylhexyl) phthalate (DEHP), alters neural function and behavior due to down-regulation of androgen receptors (AR). Testosterone signaling via AR plays a pivotal role not only in reproductive function and behavior, but also in cerebrovascular integrity. The link between reduced androgen sensitivity, blood–brain barrier (BBB) dysfunction and neuroinflammatory process has been demonstrated. Whether phthalates exposure affects the integrity and function of the BBB remained to be explored. Therefore, the impact of adult exposure to low doses of DEHP alone or in an environmental phthalate mixture on the BBB integrity and surrounding parenchyma known as neurovascular unit (NVU) was studied. For this purpose, two-month-old C57BL/6J males were orally exposed for 6 weeks to DEHP alone (0, 5, and 50 µg/kg/day) or to DEHP (5 µg/kg/day) in an environmental phthalate mixture. BBB permeability, protein levels of tight junction components, glial activation and neuroinflammation were investigated in the hypothalamic medial preoptic area (mPOA) and hippocampus, two brain regions sensitive to testosterone, involved respectively in reproductive and cognitive functions. Exposure to DEHP alone or in a phthalate mixture increased BBB permeability to exogenous and endogenous tracers and affected the accessory tight junction protein Zona Occludens-1 and caveolae protein Cav-1 in the mPOA and the hippocampal CA1 and CA3 areas. This was associated with an inflammatory profile including an astrocyte activation and induced nitric oxide synthase (iNOS) expression in the mPOA, and a microglial activation in the mPOA and the hippocampal CA1 and CA3 areas. The protein levels of the inflammatory molecule, cyclooxygenase-2 were increased in activated microglial cells of the exposed mPOA. None of the major effects induced by DEHP alone or in a mixture was detected in the hippocampal dentate gyrus. DEHP alone or in phthalates mixture decreased the AR protein levels in cerebral capillaries of the mPOA. Such exposure reduced the protein levels of basement membrane components and receptors involved in cell-matrix interactions while matrix metalloproteases MMP-2 and MMP-9 activity was enhanced. These data point out a vulnerability of the NVU to exposure to DEHP alone or in a phthalate mixture, with similarities and differences in the induced effects between the studied brain regions. Our data highlight that environmental exposure to phthalates, could alter AR / Cav-1 interaction, impacting a Cav-1 / nitric oxide / MMP pathway, thus leading to disruption of essential glio-neurovascular coupling to the functioning of neurons in the mPOA. This suggests that environmental exposure to endocrine disruptors, such as phthalates, could represent a risk factor for the cerebrovascular function. The failure of BBB functionality in sensitive brain to anti-androgenic components such as phthalates could participate, in the reported alteration of sexual and cognitive behaviors.

Keywords: Endocrine disruptors, Phthalates, Neurovascular unit, Androgen receptor, Neuroinflammation

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Abbreviations

ABC: Atp-Binding Cassette

AD: Alzheimer's Disease

AF: Activation Function

AJS: Adherens Junctions

AMT: Adsorptive- Mediated Transcytosis

AQP4: Aquaporin

ARs: Androgen Receptors

BBB: Blood-Brain Barrier

BBP: Benzylbutyl Phthalate

BCB: Blood-Cerebrospinal Fluid Barrier

BCRP: Breast Cancer Resistance Protein

BDNF: Brain-Derived Neurotrophic Factor

BPA: Bisphenol A

BW: Body Weight

CAV: Caveolin

CBF: Cerebral Blood Flow

CMT: Solute Carrier-Mediated Transport

CNS: Central Nervous System

COX: Cyclooxygenases

CSF: Cerebrospinal Fluid

CVOS: Circumventricular Organs

CX: Connexin

DBD: DNA-Binding Domain

DBP: Dibutyl Phthalate

DDT: Dichlorodiphenyltrichloroethane

DEHP: Di-2- Ethylhexyl-Phthalate

DEP: Diethyl Phthalate

DHT: Dihydrotestosterone

DIBP: Diisobutyl Phthalate

DNBP: Dibutyl Phthalate

E₂ : 17 β -estradiol

EA : Experimental Autoimmune Encephalomyelitis

ECL: Extracellular Loops

ECM: Extracellular Matrix

ECs: Endothelial Cells

EDCS: Endocrine-Disrupting Chemicals

EFSA: European Food Safety Authority

ERs: Estrogen Receptors

ESAM: Cell-Selective Adhesion Molecule

FSH: Stimulating Hormone

GFAP: Glial Fibrillary Acidic Protein

GLUT: Glucose Transporter

GPCRS: G-Protein-Coupled Receptors

GS: Glutamine Synthetase

HBGV: Health-Based Guidance Value

HPG: Hypothalamic-Pituitary-Gonadal

HSPs: Heat Shock Proteins

IBA-1: Ionized Calcium Binding Adapter Molecule 1

ICA: Internal Carotid Arteries

IFN γ , Interferon gamma

IL : Interleukin

JAM : Junction Adhesion Molecules

kDa: Kilodalton Unit

KO: Knockout

LBD: Ligand-Binding Domain

LH: Luteinizing Hormone

LOAEL: Lowest Observed Adverse Effect Level

MAGI-1: Membrane-Associated Guanylate Kinase Inverted-1

MARs: Membrane-Associated Androgen Receptors

MCA: Middle Cerebral Artery

MDR: Multi Drug Resistant

MEA: Medial Amygdala

MEHP: Mono-2-Ethylhexyl Phthalate

MIP-1A: Macrophage Inflammatory Protein-1 α

MMP: Matrix Metalloproteinase

MRPS: Multidrug Resistance-Associated Proteins

MS: Multiple Sclerosis

NADPH: Nicotinamide Adenine Dinucleotide Phosphate

NDRG2: Member 2 Of N-Myc Downstream-Regulated Genes Family

NF- κ B: Nuclear Factor κ -Light-Chain-Enhancer of Activated B Cells

NO: Nitric Oxide

NOS: Nitric Oxide Synthase

NRP : Neuropilin

NTD : N-terminal Domain

NVU : Neurovascular Unit

OAT3 : Organic Anion Transporter 3

OXPHOS : Oxidative Phosphorylation

PAG : Phosphate Activated Glutaminase

PAHS : Polycyclic Aromatic Hydrocarbons

PCBS : Polychlorinated Biphenyls

PD: Parkinson's Diseases

P-GP: P-Glycoprotein

PKA: Protein Kinase A

POA: Preoptic Region

PVC: Polyvinylchloride

RMT: Receptor-Mediated Transcytosis

RNS: Reactive Nitrogen Species

RNS: Reactive Nitrogen Species

ROS: Reactive Oxygen Species

RTK: Receptor Tyrosine Kinase

S100 β : Calcium Binding Protein Beta

SLC: Solute Carrier Transporter

TCA: Tricarboxylic Acid

TDI: The tolerable daily intake dose

TET2: Ten-Eleven Translocation 2

TGF- β : Transforming Growth Factor-Beta

TJS: Tight Junctions

TLR4: Toll-Like Receptor-4

TNF- α : Tumor Necrosis Factor Alpha

TR4: Testicular Receptor 4

TREM2: Triggering Receptors Expressed On Myeloid Cells-2

WHO: World Health Organization

ZOs: Zonula Occludens

1 Chapter 1: Blood-brain barrier and neurovascular unit

1.1 Cerebral vascular anatomy and physiology

The central nervous system (CNS) consists of the brain and spinal cord that are strategically placed and architecturally unique, thus representing a functionally integrated regulatory complex. The brain constitutes only 2% of the total body mass while it consumes 20% of the body's energy. The brain needs a constant blood flow and is very sensitive to its decrease (Abbott et al., 2010). In adult, the cerebral blood flow (CBF) is about 15% (750 mL per minute) of the cardiac output (Ito et al., 2005).

Arteries deliver oxygenated blood, glucose and other nutrients to the brain. Arterial blood enters the cranial cavity, primarily through two sets of blood vessels: the internal carotid arteries (ICA), which give rise to anterior brain circulation and the vertebral arteries which give rise to posterior circulation (Iadecola, 2017; Mastorakos and McGavern, 2019). The anatomy of the cerebrovascular tree is summarized in **(Fig.1)**. The ICA enters the skull and merges with branches of the vertebral arteries to form the circle of Willis at the base of the brain. The middle cerebral artery (MCA) takes off from the circle of Willis and supplies a large territory of the cerebral cortex, giving rise to pial arteries and arterioles that run on the surface of the brain, forming a heavily interconnected network from which arterioles penetrating into the substance of the brain originate the penetrating arterioles (Iadecola, 2017). The penetrating arteries go further down into the brain parenchyma, giving rise to parenchymal arterioles, which eventually branch off into capillaries.

Whereas pial and penetrating arteries are covered by vascular smooth muscle cells and are separated from brain tissues by the parenchymal basement membrane (glia limitans), parenchymal arterioles and capillaries become associated with neurons and astrocytes (Yamazaki and Kanekiyo, 2017).

Neurons within the CNS communicate using a combination of chemical and electrical signals, and precise regulation of the local ionic microenvironment around synapses and axons is critical for reliable neural signaling. It has been argued that the function of cerebrovascular

network is one of the most important function for maintaining the homeostasis of the neural microenvironment (Abbott et al., 2010).

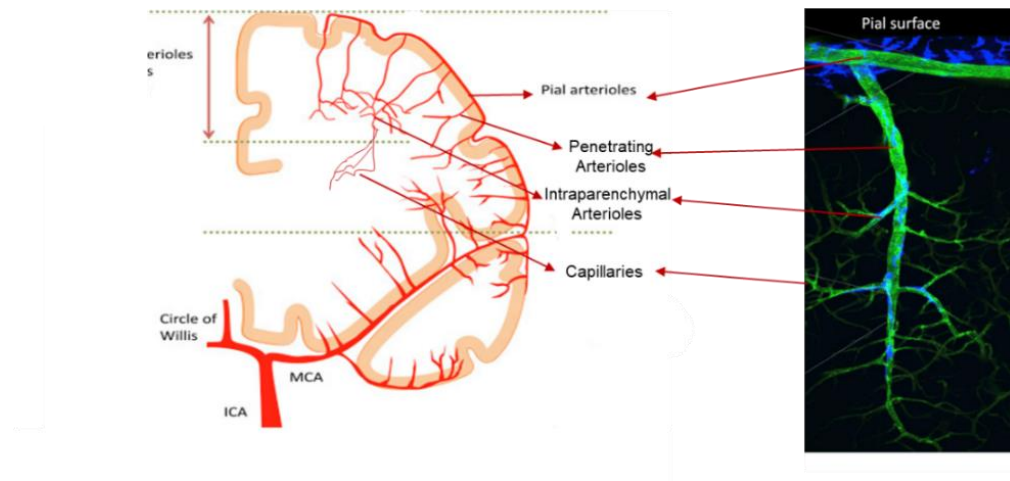


Figure 1. Anatomy of the cerebrovascular tree

ICA: Internal Carotid Artery. MCA: Middle Cerebral Artery. Figure adapted and modified from (Iadecola, 2017).

Homeostasis refers to the ability of an organism to maintain the internal environment of the body within limits, consistent with survival of the organism. It involves a self-regulating processes that return critical systems of the body to a set point within a narrow range of operation, that allow an organism to survive (McEwen, 2016). Indeed, blood-brain exchange, which is narrowly regulated thanks to the presence of certain barrier, plays a major role in the regulation of brain hemostasis. The endothelial cells (ECs) which constitute the walls of the brain capillaries, form the largest interface for blood-brain exchange. This area depending on the anatomical area is between 150 and 200 cm²/g tissue giving a total area for exchange in the brain between 12 and 18 m² for the average human adult (Abbott et al., 2010).

1.2 Brain barriers

In the brain, the appropriate stable environment required is maintained by the “gateway” called the brain barriers (Chen et al., 2019). Three barriers’ structures limit and regulate molecular

exchanges at the interfaces between the blood and the neural tissue or its fluid spaces: the blood-cerebrospinal fluid barrier (BCB), the arachnoid barrier and the blood-brain barrier (BBB) (**fig.2**).

The cerebral ventricles and subarachnoid space contain cerebrospinal fluid (CSF), which is secreted by choroid plexuses in the lateral, third and fourth ventricles (Abbott et al., 2006). The arachnoid barrier consists of the arachnoid mater which is an epithelial layer that provides a barrier between the peripheral vasculature of the dura mater and the subarachnoid CSF through tight junctions and efflux pumps (Mastorakos and McGavern, 2019).

The BCB represents a barrier between the circulating blood and the CSF space. The BCB is formed by epithelial cells of the choroid plexuses located in the four ventricles of the brain and the subarachnoid epithelial structures facing the CSF space in intracranial and spinal areas (Tumani et al., 2018).

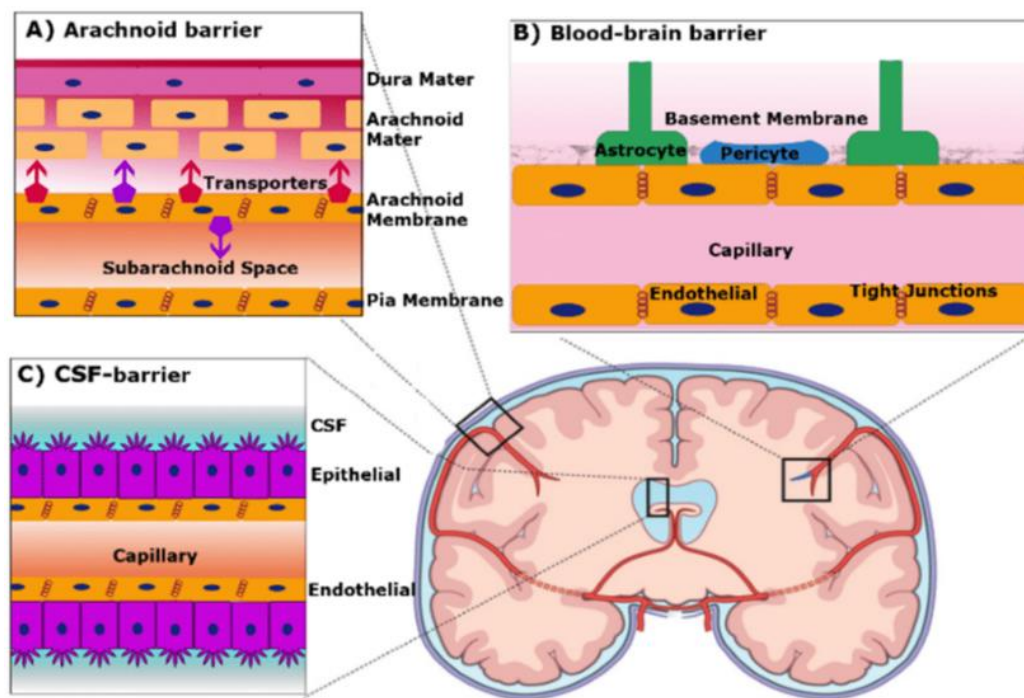


Figure 2. Different barriers of the brain

Detailing the different barriers of the brain. A) The Arachnoid barrier, showing efflux pump transporters and ECs. B) the BBB formed by the cerebrovascular ECs between blood and brain interstitial fluid C) The BCB is composed of epithelial and ECs. Adapted from (McCully et al., 2018).

One of the most important and specialized functions of the brain microvasculature is the BBB, which is formed by the cerebrovascular ECs between blood and brain interstitial fluid (Yamazaki and Kanekiyo, 2017). The cerebral microvasculature consists in a dense network of capillaries, arterioles and venules, although to a different extent all of which retain BBB properties. The BBB not only restricts the free transport of different potentially harmful substances to the brain, but plays a critical role in the transport of nutrients to the brain and removal of metabolites (Wilhelm et al., 2016).

These mentioned-barriers are important for the maintenance of brain homeostasis, whereas they differ in their morphology and physiology. For example, the BBB is sealed by tight junctions and does not show any permanent fenestration while the BCB has several fenestrations (gap junctions) and pinocytosis vesicles (Tumani et al., 2018). For more information about the structural variations in the anatomy of cerebrovascular barriers, the readers are referred to (Mastorakos and McGavern, 2019).

Among the mentioned-barriers, the BBB has received more attention regarding CNS pathology, nevertheless, there is a growing body of evidence showing that the BCB barrier plays a crucial role in the spread of inflammatory mediators from the periphery to the CNS and contributes to the pathogenesis and progression of various neurological disorders (Solár et al., 2020).

1.3 Circumventricular organs

Circumventricular organs (CVOs) are structures around the third and fourth brain ventricles that are in contact with blood and CSF. CVOs are characterized by their extensive and highly permeable capillaries unlike those in the rest of the brain where there exists a BBB at the capillary level (Prevot et al., 2018). The leaky endothelium in the CVOs which contain neurons specialized for neurosecretion and/or chemosensitivity, allows tissue–blood exchange (Abbott et al., 2006). The CVOs include the subfornical organ, the area postrema , the vascular organ of lamina terminalis, the median eminence, the pituitary neural lobe, and the pineal gland (Prevot et al., 2018). The interface ensuring the cerebral homeostasis, in these cerebral areas, is constituted by

specific cells called tanycytes, lining cerebral ventricles, which are sealed by tight junctions. Indeed, tanycyte-like cells form a BCB barrier in the CVOs regions (Langlet et al., 2013).

1.4 History of the Blood-Brain Barrier

The original concept of a BBB is often attributed to Paul Ehrlich (Ehrlich, 1885). Basis of his experiments with dye, he observed that most acidic dyes, including trypan blue, injected into blood stream readily stain most regions of the body but not the brain and spinal cord; however, he did not accept that permeability of cerebral vessels was different from other organs and concluded that it was due to the low affinity of CNS for these dyes. Goldmann, in 1913, is often credited with the first experiments showing injection of the same dyes into the subarachnoid space of rats readily stained nervous tissue, but not the rest of body's organs (Goldmann, 1913). This finding falsified the hypothesis that the differential staining was due to lower affinity for the dyes in the CNS tissue, suggesting that once within the CNS, the dyes did not cross the wall of brain capillaries to the blood circulation (Saunders et al., 2014). However, the earlier experiments of Bouffard in 1906 and of Franke in 1905 who showed that methylene blue and trypan red stained all tissues except the brain, are rarely cited. None of these authors did not offer an explanation of the lack of staining of the brain by the different dyes (see for review, Saunders et al., 2014).

The term “blood-brain barrier” “Blut-Hirnschranke” is attributed to Lewandowsky in 1900. Indeed, Lewandowsky clearly proposed that cerebral capillaries have specific restrictive properties with respect to some compounds but it does not appear in his papers. The first person to use this term seems to be Stern and Gautier in the early 1918 in the paper entitled “The barrier which opposes the movement into the CSF of substances in the blood shows notable differences in different animal species” (see for review, Saunders et al., 2014). A great review was attempted to clarify history of the concept of blood brain barrier by a careful translation of original papers, some published a century ago, as well as providing discussion of studies claiming the existence of the BBB (see for review, Saunders et al., 2014).

The properties of the cerebral vasculature remained unknown due to the limited resolution of the light microscope, then discovery of electron microscopy allowed to define the ultrastructural features of blood vessels throughout the body. Early ultrastructural investigation (1955-1959) questioned the selective role of ECs in the barrier function as well as its surrounding cells (Ribatti et al., 2006).

1.5 Structure and function of the blood-brain barrier

As already mentioned the CNS cells require a stable microenvironment, which is guaranteed by a cellular and molecular complex system involving brain microvessels and their close environment referred to as the BBB (**Fig.3**) (Abbott et al., 2010; Herndon et al., 2017). The function of the BBB as a highly regulated specific interface underlies the regulation of all vital functions of an organism by maintaining cerebral homeostasis to ensure neuronal activity.

The BBB is formed by ECs which are themselves sealed by tight junctions (TJs) and adherens junctions (AJs) (Abbott et al., 2010; Weiss et al., 2009), thereby limiting the paracellular diffusion of substances. The major transmembrane components of TJs are the claudins, occludin and junction adhesion molecules (JAMs) (Blanchette and Daneman, 2015). They interact with the actin cytoskeleton via cytoplasmic TJs accessory proteins (cytoplasmic scaffolding proteins) including zonula occludens (ZO) proteins (Keaney and Campbell, 2015). The AJs transmembrane components cadherin and nectin anchoring to cytoplasmic components such as catenin and afadin respectively, are also involved in the BBB integrity (Chow and Gu, 2015; Tietz and Engelhardt, 2015).

The BBB also provides a gate-like function for substance delivery to brain cells by limiting trans-cellular diffusion via selective carrier-mediated transport systems (Abbott et al., 2010) present in EC abluminal or luminal plasma membranes. As such, the ability of macromolecules, fluids, and cells to cross endothelial barriers depends on charge, size, and binding characteristics (Azzi et al., 2013). 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) (Girardin, 2006). Notably, efflux transporters (e.g., P-glycoprotein; P-gp, Breast Cancer Resistance Protein; BCRB and the Organic Anion Transporter 3; OAT3) are also responsible for the transport of several classes of pharmaceutical drugs (Choudhuri and Klaassen, 2006; Liu and Liu, 2019). The efflux transporters also present a major obstacle in central distribution of many therapeutic agents that are beneficial to treat CNS diseases. A numerous of lipid-soluble molecules such as some therapeutic drugs are the substrates for the efflux transporters. Some efflux transporters limit brain uptake of these agents in order to protect the brain from potentially harmful exogenous substances (Choudhuri and Klaassen, 2006; Löscher and Potschka, 2005).

An extremely low rate of transendothelial vesicular transport also integrates the transcellular pathway to minimize the uptake of substances from blood to brain parenchyma (Villaseñor et al., 2019). Among the different categories of vesicular transport, caveolae appears to be the major element responsible for transcytosis in cerebral endothelial cells (Villaseñor et al., 2019).

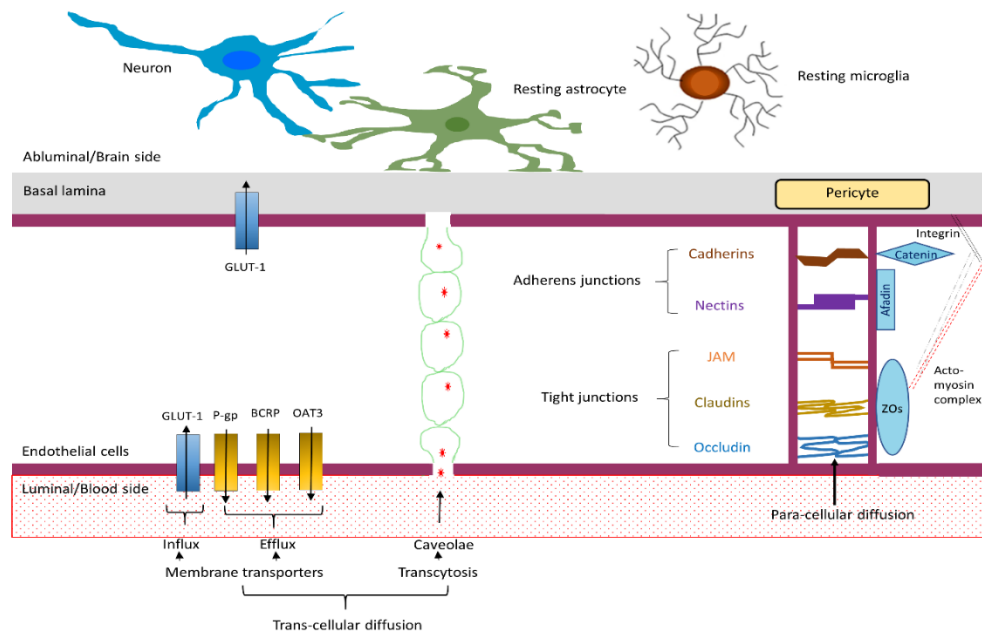


Figure 3. The major cellular elements of BBB localized at CNS

Micro-vessels are comprised of a continuous layer of ECs, 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; ZO, zonula occludens proteins. adapted from (Ahmadpour and Grange-Messent, 2021).

Both of tight paracellular and transcellular barriers create a polarized cell with distinct luminal and abluminal membrane compartments such that movement between the blood and the brain can be tightly controlled through regulated cellular transport properties (Daneman and Prat, 2015). The ability of a single layer of cells to transport solutes from one side of the cell to the other against a concentration gradient is seen in many epithelia such as those lining kidney tubules and intestinal mucosa. Active transcellular transport in these cells is thought to be due to a selective or polar distribution of transport proteins between the opposite surfaces of the cell (Betz et al., 1980). As an example, a solute can be greatly accumulated within the cell by active transport across one membrane and subsequently leave the cell by an alternate nonactive process present in the opposite membrane. Therefore, cellular polarity permits vectorial transport against concentration gradients and across cellular barriers (Betz et al., 1980). The two opposing plasma membranes of the endothelial cell do not need have identical permeability properties. Thus, a comprehensive representation of the BBB must take into account the two

separate membranes that solutes encounter as they move between blood and brain as well as the cytoplasm of the endothelial cell which may contain enzymes capable of reacting with the solutes while they are in transit through the cell (Betz, 1989). Generally, lipophilic/hydrophobic substances can cross the BBB passively (e.g., O₂, CO₂, ethanol, caffeine, nicotine), whereas lipophobic/hydrophilic/large molecules substances cannot. Specific transporters are present in endothelial cells to ensure sufficient glucose and amino acid transmission. This allows the BBB to tightly regulate CNS homeostasis as well as protect the CNS from toxins, pathogens, inflammation, injury and disease (Shah and Jeyaretna, 2018). As such, BBB dysfunction can lead to many brain diseases as well as the complication of recovery from injuries and insults (Abbott et al., 2010; Archer and Ravussin, 1994).

The BBB represents a complex cellular system that consists not only of ECs, but also of pericytes embedded in basement membrane surrounding ECs, and astrocytic end-feet anchored to this basement membrane. ECs, pericytes, basement membrane, astrocytic end-feet and their neighboring microglial cells and neurons are collectively named the neurovascular unit (NVU) (**Fig.3**); they govern BBB development, along with its maintenance and integrity (Erdő et al., 2017).

Glial cells including microglial and astrocytes represent important elements of the NVU. Microglial cells are activated during inflammatory reaction or immune challenge in the CNS and then differentiate into resident CNS macrophages (Johann and Beyer, 2013). Upon immune challenge or following microglia activation, astrocytes also produce inflammatory cytokines (Cabezas et al., 2014; Sofroniew and Vinters, 2010). The presence of glial cells in the vicinity of the BBB promotes NVU susceptibility to neuroinflammatory responses. Neuroinflammation, in turn, can contribute to BBB dysfunction and neurodegenerative process (Vodo et al., 2013).

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 (Witt and Sandoval, 2014). Abnormal NVU function is a wide-range of neurodegenerative and neuropsychiatric diseases (Hawkins, 2005), as well as in physiological aging (Erdő et al., 2017).

1.5.1 Endothelial cells

Capillaries have a diameter of 5-10 μm and are lined by a single layer of EC. ECs across organs are classified as continuous, fenestrated and discontinuous (sinusoidal capillaries) based on their morphology (**Fig.4**). These different types of ECs confer varying degrees of molecular exchange between the blood and the tissue. Discontinuous ECs have large intercellular gaps and fenestrated endothelial cells have windows or pores. On the other hands, continuous ECs are the least permissive of these three types thanks to continuous intercellular molecular binding systems between cells. Continuous endothelium is found in many tissues including the lung, muscle and the brain. They exist in a more specialized state in the CNS (Augustin and Koh, 2017). The walls of cerebral blood vessels are formed of ECs that are sealed by continuous intercellular molecular binding systems (Erdő et al., 2017).

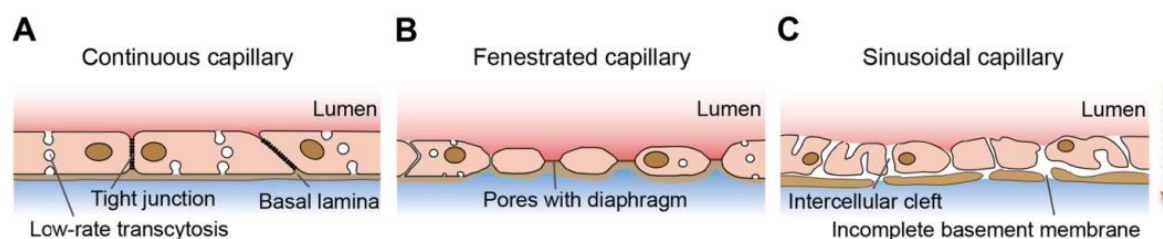


Figure 4. Three major types of capillaries

Three major and two specialized types of capillaries. (A) Barrier-forming continuous capillaries are found in most organs including brain and retina (B) Fenestrated capillaries have intracellular pores and are covered with a diaphragm; this structure is found in endocrine glands, intestine and kidney, rendering them permeable to fluid and small molecules. (C) Sinusoidal (discontinuous) capillaries have larger intercellular gaps and a discontinuous basement membrane allowing free exchange of materials; this structure is generally found in liver, bone marrow and spleen. Adapted from (Augustin and Koh, 2017).

1.5.2 Intercellular molecular binding systems

The four types of cell–cell junctions present in vertebrates are TJs, AJs, gap junctions and desmosomes (Feldman et al., 2005). In endothelia, the continuous intercellular molecular binding systems TJs and AJs (Erdő et al., 2017) that limit the transfer of ions, water and substances through the intercellular space between the cells, refer to the paracellular diffusion (Weiss et al., 2009). In addition, the crosstalk between TJs and AJs, play an important role in the maintaining

of barrier integrity (Tietz and Engelhardt, 2015). Abnormal cerebrovascular TJs or AJs have been detected in a wide-range of CNS diseases (Hawkins, 2005).

1.5.2.1 Tight junctions

The main structures responsible for the barrier properties are the TJs. Each TJ component has a specific role and provides a vital and important contribution to overall BBB integrity. Damage to or loss of TJs results in a compromised BBB and progression of brain diseases (Greene and Campbell, 2016; Wolburg and Lippoldt, 2002).

Occludin was the first tight junctional transmembrane molecule discovered. The barrier function of occludin depends on its special structure domains (Wolburg and Lippoldt, 2002). Occludin is a 65 kDa integral membrane protein with 502 amino-acid residues, which includes two extracellular loops (ECL), one intracellular loop, and its carboxyl and amino-terminals oriented toward the cytoplasm (**Fig.5**). One of the extracellular loops (ECL1) of occludin has a very high content of tyrosine and glycine residues. The tyrosine residues are involved in forming hydrophobic interactions and H-bonds, while glycine residues provide flexibility. The other extracellular loop (ECL2) is rich in tyrosine residues and contains two cysteines to form disulfide bridges in the oxidizing environment, which is sensitive to hypoxia and occurs homo-oligomerization. ECL2 serves as the main binding domain, which interacts with other TJs and regulates the function of other TJs. In addition, C-terminal cytoplasmic of occludin is rich in serine, threonine, and tyrosine residues and directly connects to ZO-1 and actin cytoskeleton. Both external loops as well as the transmembrane and the C-terminal cytoplasmic domains of occludin, are important for the regulation of paracellular permeability between adjacent cells (Yuan et al., 2020).

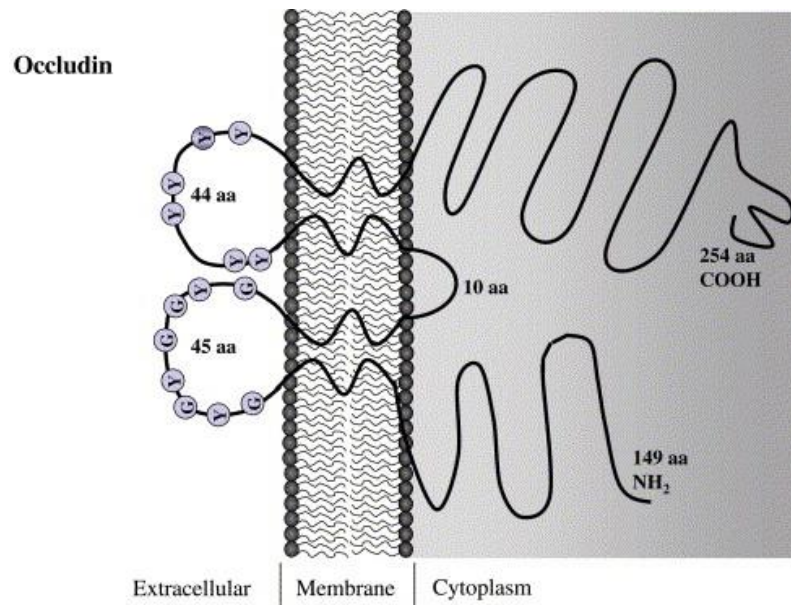


Figure 5. Schematic representation of occludin structure

aa: amino-acid. Y : Tyrosine. G : glycine. Adapted from (Feldman et al., 2005).

Several binding partners of occludin have been determined and this increasing knowledge of the molecular interactions of occludin has aided the study of its precise functions. A number of TJ proteins and TJ-associated proteins have been shown to co-localize or interact with occludin. ZO-1 and ZO-2 were the first junctional components known to directly interact with occludin. To more information regarding the current knowledge of interaction of occludin with TJ associated proteins and the binding partners, the reader can refer to a great review in references (Feldman et al., 2005).

It has been shown that occludin is pivotal for TJ formation and stability of the TJ assembly (Greene and Campbell, 2016). Several studies have reported that the alteration or decrease in occludin during cerebral ischemia results in increased BBB permeability. It has been demonstrated that autophagy contributes to BBB disruption during ischemia through occludin degradation (Kim et al., 2020). *In vivo* and *in vitro* evidences show that an increase of active matrix metalloproteinase (MMP) MMP-2 and MMP-9 mediate occludin degradation in pathological conditions (Yuan et al., 2020). Thus MMP-mediated occludin degradation has been reported as a potential pathway for TJ dysfunction.

Claudins constitute a large family of 20-27 kDa membrane proteins with four transmembrane domains, expressed in TJs in various cell types. Claudins support TJ integrity via their capacity of cis- and trans-homodimerization as well as heterodimerization, notably through their second ECL, as recently reported for claudin-5 (Luissint et al., 2012). Claudin-5 consists of 4 transmembrane domains, a short NH₂ terminus, two ECL, a short intracellular loop and a longer COOH terminus (**Fig.6**). ECL1 contains a disulphide bond and ion binding site as well as a highly conserved signature motif. The long COOH terminus contains the PDZ binding motif for interactions with scaffolding proteins including ZO-1, -2 and -3. Additionally, the COOH terminus contains trafficking and phosphorylation residues (Greene et al., 2019).

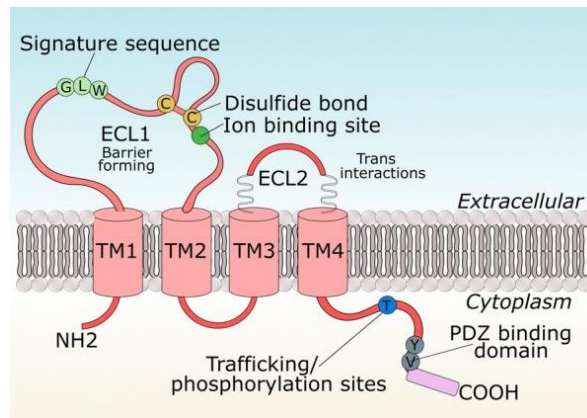


Figure 6. Schematic representation of claudin-5 structure

Claudin-5 consists of 4 transmembrane domains (TM), a short NH₂ terminus, two extracellular loops (ECL), a short intracellular loop and a longer COOH terminus. ECL1 contains a disulphide bond and ion binding site as well as a highly conserved signature motif. The long COOH terminus contains the PDZ binding motif for interactions with scaffolding proteins including ZO-1, -2 and -3. Additionally, the COOH terminus contains trafficking and phosphorylation residues. Adapted from (Greene et al., 2019).

It has been reported that brain endothelial cells predominantly express claudin-3, claudin-5 and claudin-12 (Luissint et al., 2012).

The presence of claudin-3 in TJs of mouse BBB endothelial cells has recently call into question. Recent data suggest that the previous detection of claudin-3 in mouse BBB endothelial cells is due to the cross-reactivity of claudin-3 antibodies to an undisclosed endothelial junctional antigen still present in claudin-3 deficient mice (Dias et al., 2019). C57BL/6J mice lacking claudin-

3 expression displayed an intact BBB and did not show any signs of BBB dysfunction (Dias et al., 2019).

Claudin-5 knockout mice, however, have a significant paracellular ionic selectivity defect (Jia et al., 2014; Yang and Rosenberg, 2011). An exogenous expression of claudin-5 strengthens barrier properties in cultured rat brain endothelial cells, whereas depletion of claudin-5 induces the disruption of the BBB in genetically-altered mice and in cultured human brain endothelial cells. Claudin-5 controls paracellular permeation of small molecules (smaller than 800 kDa), induces and maintains tightness of TJs (Haseloff et al., 2015). A review published early in 2019 provides a comprehensive overview of the role of claudin-5 and its regulation in physiological and pathological conditions (Greene et al., 2019).

Claudin-12 is an atypical claudin and has been barely investigated. Its second extracellular loop is extended, and both extracellular loops exhibit low levels of homology to the other BBB claudins. Claudin-12 lacks a C-terminal PDZ-binding motif. The exact function of claudin-12 at the BBB and at other barriers is unknown. Recent investigation revealed that claudin-12 is not essential in establishing or maintaining BBB TJs integrity. Claudin-12 is rather expressed in cells that typically lack TJs suggesting that claudin-12 plays a role other than forming classical TJs (Castro Dias et al., 2019). Analysis of tight junction transcripts revealed that there is a consistent and robust negative correlation between the expression of TJ mRNAs and the duration of psychiatric disease with longer duration of disease associated with reduced expression of Claudin-12 (Greene et al., 2020). However in epithelial cell cultures claudin-12 knock-down decreases the effect of vitamin D-induced Ca^{2+} transfer across epithelial cell indicating its involvement in paracellular Ca^{2+} permeation (Haseloff et al., 2015).

In addition to occludin and claudins, JAMs are transmembrane proteins belonging to the cortical thymocyte marker of the *Xenopus* (CTX) family, a member of the immunoglobulin (Ig) superfamily (Luissint et al., 2012). JAMs have two extracellular Ig-like domains and cytoplasmic tail with a PDZ-binding motif. JAMs engage in homophilic and heterophilic interactions among their family members and bind integrins (Garrido-Urbani et al., 2014). The recently discovered

molecule endothelial cell-selective adhesion molecule (ESAM) are localized at tight junctions as well (Wolburg and Lippoldt, 2002).

Major members of the JAM family include: JAM-A (mainly expressed in mouse and human BBB endothelial TJs, and in epithelial cells), JAM-B and JAM-C (mainly expressed in most vascular endothelial cells, mouse BBB endothelial TJs) (Luissint et al., 2012).

JAM-A localizes in close proximity of TJ strands but not is restricted to cells that form junctions. It is also expressed in platelets and cells of the immune system. JAM-B localizes to the junctions of endothelial cells from different vessels, but mostly in high endothelial venules, which are portals of entry into lymphatic tissues for blood-borne lymphocytes responding to processed antigens. JAM-C is expressed in vascular endothelial cells, including high endothelial venules, and in lymphatic vessels but also in platelets, T cells, natural killer and dendritic cells (Bazzoni, 2003)

Unlike occludin and claudins, JAMs, however are not essential to TJ formation in epithelial and endothelial cells. They may involve in the facilitation of assembly of TJ components and in the establishment of cell polarity by recruiting the polarity complex (Luissint et al., 2012). JAM proteins are able to reduce cell permeability and increase resistance to macromolecules. In an inflammation model, a JAM-A antibody was shown to increase vascular endothelial permeability beside, It has been suggested that JAM-A play a pivotal role in controlling brain metastasis (Jia et al., 2013). Altered expression of JAM-A, in addition to affecting junctional tightness may also both reflect and affect leukocyte trafficking, with implications for immune status within the diseased CNS such as multiple sclerosis (MS) (Padden et al., 2007).

1.5.2.2 Adherens *junctions*

The adherens junctions are necessary for the primary contact between endothelial cells. This contact is made by transmembrane components such as cadherin and nectin anchoring to a group of cytoplasmic molecules such as catenins, desmoplakin or afadin (Chow and Gu, 2015; Tietz and Engelhardt, 2015; Wolburg and Lippoldt, 2002).

E-cadherin is a member of classical cadherin family (Type I) that mediating homophilic adhesions in cis and trans. It links to actin cytoskeleton via catenin complex. E-cadherin is implicated in controlling barrier integrity, junctional plasticity, and cytoskeletal tension (Lutz and Siahaan, 1997; On et al., 2014). N-cadherin is another transmembrane protein of the classical cadherin family (Type I) which is implicated in mediation of pericyte–endothelial interactions during brain angiogenesis (Perrot et al., 2020). VE-cadherin is member of the classical cadherin family (Type II). VE-cadherin is a main integral membrane protein of endothelial AJs and cytoplasmic domain links AJs via catenin complex to actin cytoskeleton and intermediate filaments (Sauteur et al., 2017). VE-cadherin is involved in homophilic cell adhesion molecule of endothelial AJs and controls endothelial cell survival, stabilization of blood vessel assembly, as well as vascular permeability (Broman et al., 2006). It was suggested that VE-cadherin can partially be a biomarker of neuroinflammation disease and lead to novel approaches for abating BBB dysregulation under pathological conditions (Li et al., 2018b).

Nectin is a member of the nectin family of the Ig superfamily that are composed of four members (nectin-1, -2, -3, and -4) that mediate cell–cell adhesion. In addition to cell–cell adhesion, nectins regulate a variety of cell functions, including cell migration polarization, differentiation, proliferation, and survival (Tietz and Engelhardt, 2015). Nectins have highly conserved cytoplasmic tail binds to the adaptor protein afadin linking nectins to actin cytoskeleton (Takai and Nakanishi, 2003). In addition, nectin–afadin complex is involved in the formation of AJs and TJs. Nectin controls VE-cadherin phosphorylation and thus vascular junctional integrity and leukocyte diapedesis (Tietz and Engelhardt, 2015). Results of Miyata et al uncovered for the first time the localization and critical functions of nectin-2 in the brain (Miyata et al., 2016). They observed that the genetic ablation of nectin-2 can caused degeneration of astrocytic perivascular end-foot processes and neurons in the cerebral cortex (Miyata et al., 2016). It was shown that the breakdown of the AJs in the gut and vascular barrier in patients with deficit schizophrenia lead to the complicity of the disease (Maes et al., 2019).

1.5.2.3 Transmembrane proteins outside of junction complex

Transmembrane proteins outside of organized TJs and AJs include PECAM-1 (also known as CD31), CD99 and CD99L (Tietz and Engelhardt, 2015). PECAM-1 contribute to barrier-enhancing properties and influence ECs barrier function by cross-talk with other junctional molecules (Privratsky and Newman, 2014), while CD99 and CD99L have shown implicated in T cell recruitment across the brain endothelium (Bixel et al., 2004).

1.5.2.4 *Cytoplasmic scaffolding proteins of the tight junction*

Transmembrane components of TJs interact with the actin cytoskeleton via cytoplasmic scaffolding proteins. A number of cytoplasmic proteins have been described to be associated with TJ transmembrane proteins and to contribute somehow to TJ integrity in epithelial cells and in brain endothelial cells. Among them, the PDZ domain-containing membrane-associated guanylate kinase (MAGUK) family members have been largely documented: zonula occludens-1 (ZO-1, 225 kDa), ZO-2 (160 kDa), and ZO-3 (130 kDa) (**Fig.7**) (Luissint et al., 2012). The MAGUK proteins ZO-1, ZO-2, and ZO-3 are associated directly with the carboxy terminus of occludin, claudins, and JAMs, but also form independent complexes themselves, forming heterodimers (ZO-1/ZO-3; ZO-1/ZO-2) through interactions of their PDZ domains (Bauer et al., 2004). It is well established that ZO proteins are essential to the assembly of claudins, occludin and JAM-A at TJs, then anchoring this multimolecular complex to the actin cytoskeleton (Luissint et al., 2012).

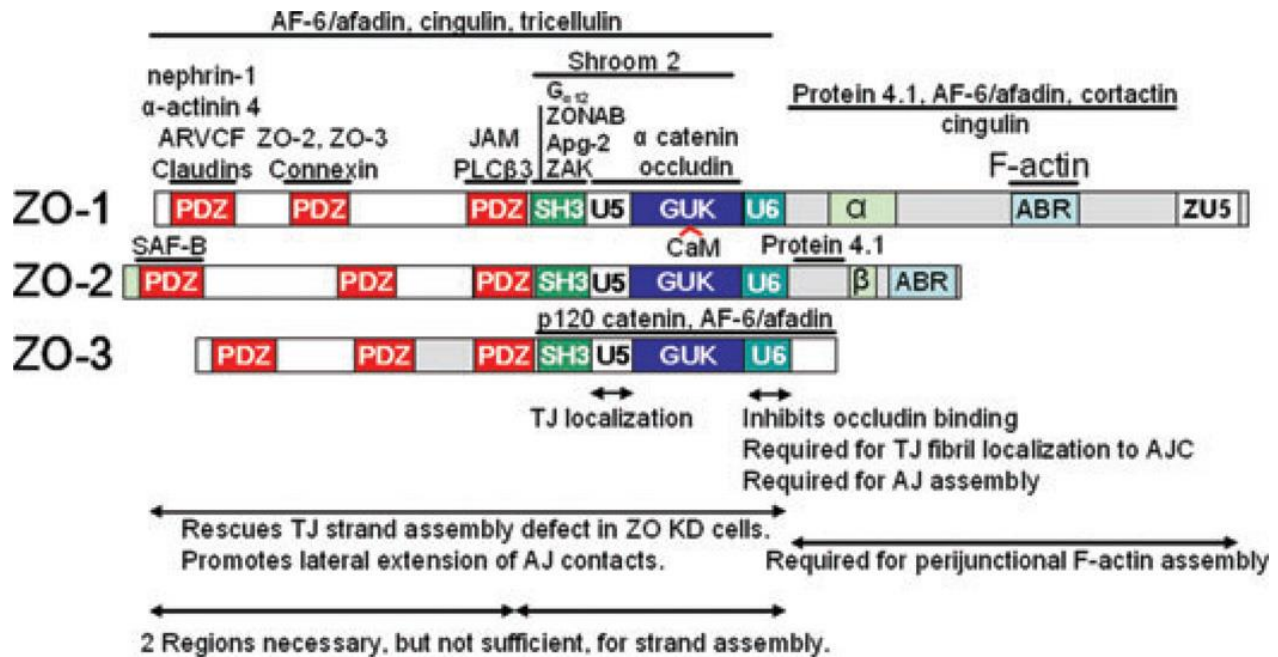


Figure 7. Protein ligands and their binding sites on ZO-1, ZO-2 and ZO-3

Protein ligands and their binding sites on ZO-1 (top), ZO-2 (middle), and ZO-3 (bottom). ABR, actin-binding region; ARVCF, armadillo repeat gene deleted in velo-cardiofacial syndrome; CaM, calmodulin; GUK, guanylate kinase-like domain; PDZ, PSD-95/discs-large/zonula occludens-1 domain; PLCB3, phospholipase-C B3; SAF-B, scaffold attachment factor-B; SH3, Src Homology-3 domain; ZAK, zonula occludens kinase; ZONAB, ZO-1-associated nucleic acid binding protein; α, β, and ZU5, alternatively spliced domains of unknown function. Adapted from (Fanning and Anderson, 2009).

ZO-1 represents the first protein positively associated with the TJ and is one of the best studied MAGUK family member. ZO-1 serves as recognition protein for TJ placement and as support for signal transduction proteins, and the loss of ZO-1 could result in a disorganization of TJ (Abdullahi et al., 2018). Disruption of ZO-1 function is the major reason of edema and hemorrhagic transformation after ischemic stroke (Jiao et al., 2011).

Other scaffolding proteins of the TJs include but not limit to cingulin and cingulin-like protein 1, afadin, MAGI-1 (membrane-associated guanylate kinase inverted-1) and MAGI-3 and MPDZ (Multiple PDZ domain protein, aka MUPP-1) (Van Itallie and Anderson, 2014).

It is important to note that scaffolding proteins are associated not only to the TJs but also with AJs (Itoh et al., 1993, 1999). Most of the signaling pathways involved in TJs and AJs regulation

including G-proteins, serine-, threonine- and tyrosine-kinases, extra and intracellular calcium levels, cAMP levels, proteases and cytokines, mediate their action via the modulation of cytoskeletal elements and scaffolding proteins connection to the cytoskeleton (Wolburg and Lippoldt, 2002).

1.5.3 Cytoskeleton

An intriguing feature of eukaryotic cells is the ability of extracts that contain cytosol, devoid of organelles, to roughly maintain the shape of the cell and even to move or contract, depending on how the extracts are prepared. This maintenance of structure by the cytosol arises from a complex network of protein filaments that traverse the cell cytoplasm, called the cytoskeleton (Fletcher and Mullins, 2010). Actin, non-muscle myosin 2A, 2B, 2C and microtubules all clearly play critical roles in tight junction architecture and physiology. Cytoskeletal connections scaffold the forming contacts, provide stability against shear and serve as a matrix for signaling pathways (Van Itallie and Anderson, 2014). The actin cytoskeleton and TJs are physically linked and cytoskeletal changes may impact BBB function without the overt loss of TJ proteins (Shi et al., 2016a). As already mentioned, the TJ proteins occludin and claudin and the AJ protein cadherin are anchored to the actin cytoskeleton by multiple accessory proteins. Dynamic interactions between the cytoskeleton and TJs are therefore important for BBB maintenance. Following exposure to specific stress such as hypoxia, free radicals, cytokines and chemokines, the actin that is normally distributed throughout the EC as short filaments and diffuse monomers is polymerized into linear fibers across the EC interior. This polymerization is accompanied by acto-myosin contraction and increased cytoskeletal tension, resulting in contracted cell morphology, an impaired junctional sealing and, eventually, hyper-permeability (Shi et al., 2016b).

1.5.4 Basement membrane

The extracellular matrix (ECM) as an acellular membrane surrounding ECs, has a pivotal role in BBB integrity (Baeten and Akassoglou, 2011). The functional attributes of the ECM in the adult CNS arise directly from its composition of molecules, which are localized to three principal compartments (**Fig.8**): The basement membrane (basal lamina), the perineuronal nets and the

neural interstitial matrix (a term used to define ECM molecules that are dispersed in the parenchyma (Lau et al., 2013)).

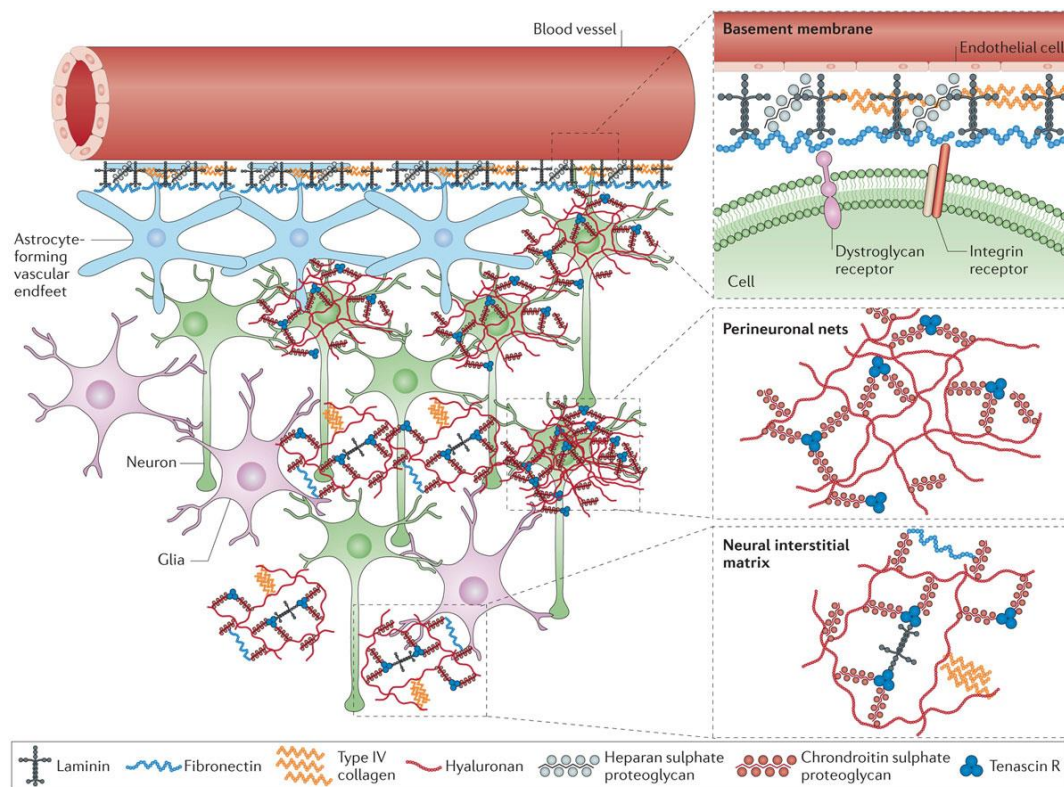


Figure 8. The three major compartments of the ECM in the CNS

Extracellular matrix (ECM) components are arranged into basement membranes that lie outside cerebral vessels, condensed as perineuronal nets around the cell bodies and dendrites of neurons or diffusely distributed as the neural interstitial matrix between cells of the CNS parenchyma. The pink glial cells depict astrocytes, oligodendrocytes or microglia. Adapted from (Lau et al., 2013).

The basement membrane, sheet-like layer, represents a non-cellular component of the NVU that largely understudied due to its intrinsic complexity and the lack of research tools. The basement membrane is a unique form of the ECM found predominantly underneath endothelial and epithelial cells. Structurally the basement membrane forms a continuous network of ECM with a thickness of 50–100 nm, composed of type IV collagen, laminin, nidogen, also known as entactin, heparan sulfate, elastin, and various proteoglycans and glycoproteins (Engelhardt and Sorokin, 2009). By contrast, the perineuronal nets, which surround neurons and synapses, comprise a condensed layer of mesh-like matrix composed principally of proteoglycans, tenascin R and link proteins that surrounds neuronal cell bodies and proximal dendrites. Besides a

probable role in preserving neuronal health, these nets are thought to be critical for maintaining synaptic plasticity. The neural interstitial matrix which fills the space between cells, consists of ECM components in the parenchyma that are not tightly associated in basement membranes or perineuronal nets; indeed, it comprises a dense network of proteoglycans, hyaluronan, tenascins and link proteins, as well as relatively small amounts of fibrous proteins (collagens and elastin) and adhesive glycoproteins (laminins and fibronectin) (Lau et al., 2013).

These ECM proteins of basement membrane are synthesized predominantly by ECs (Kramer et al., 1984), astrocytes (Bernstein et al., 1985) and pericytes (Armulik et al., 2011). In the brain, two types of basement membrane are found: an endothelial and a parenchymal basement membrane which are separated by pericytes (Rosenberg, 2017).

Collagen IV, the most abundant component of the basement membrane, is a trimeric protein containing three α -chains. Currently, six collagen IV α -chains (COL4A1–6) have been identified. Unlike COL4A3–6, which are more spatially and temporally restricted, COL4A1 and COL4A2 are present in almost all basement membrane and are highly conserved across species (Candiello et al., 2010; Xu et al., 2018).

Laminin is a T-shaped or cruciform-shaped trimeric protein composed of α , β and γ chains. So far, five α , four β and three γ chains have been identified. Various combinations of these subunits generate a large number of laminin isoforms (Yao, 2017). Although ECs, pericytes and astrocytes both make laminin at the BBB, they synthesize different laminin isoforms. For example, ECs generate laminin- $\alpha4\beta1\gamma1$ (-411) and laminin-511. Astrocytes predominately synthesize laminin-211 whereas pericytes mainly synthesize laminins containing $\alpha4$, $\alpha5$ and $\gamma1$. Due to this cell-specific expression pattern, laminin shows differential distribution between endothelial and parenchymal basement membrane. Specifically, astrocyte-derived laminin-211 is predominantly found in parenchymal, whereas endothelial cell-derived laminin-411 and laminin-511 are mainly located in endothelial compartment (Engelhardt and Sorokin, 2009). Besides, the vascular and parenchymal compartment have a different composition, for instance, the former is made up of laminins $\alpha4$ and $\alpha5$, whereas the latter contains laminins $\alpha1$ and $\alpha2$.

The commonly accepted model proposes that two networks of polymerized laminins and crosslinked collagen IV constitute the basic frame-work of every basement membrane. The importance of basement membrane proteins in BBB integrity is studied by a variety of deletion or mutations protein result in different phenotype and consequences including in embryonic or postnatally lethality, vascular disruption, BBB breakdown and intracerebral hemorrhage (Candiello et al., 2010; Xu et al., 2018). The functional significance of each basement membrane component in BBB integrity is revised meticulously in reference (Xu et al., 2018) The interaction between endothelial integrin, laminin, and other ECM proteins allows ECs to be anchored onto this basement membrane (Hawkins, 2005). In addition of its role in structural support and cell anchoring It exerts also an important functions in signaling transduction (Wang and Shuaib, 2007).

It is well established that human basement membrane undergoes age-related structural and compositional changes and that these changes are only minor or even undetectable in most laboratory animals. It has been shown that aging induces an increase of relative concentrations of collagen IV and a decrease of the laminin concentration (Candiello et al., 2010). Basement membrane provides an anchor for many signaling processes at the vasculature, but also provide an additional barrier for molecules and cells to cross before accessing the neural tissue. The dynamic nature of the ECM depends on constitutive remodeling by several protease families, including the MMPs. The MMPs have been shown to mediate the degradation of the ECM around cerebral blood vessels and subsequently leads to the BBB opening (Thomsen et al., 2017). Disruption of the basement membrane composition by MMPs is an important component in leukocyte infiltration that is observed in many different neurological disorders (Daneman and Prat, 2015).

1.5.5 Pericytes

Pericytes cover a large part of the abluminal surface (between 22% and 99% (Herndon et al., 2017) of the endothelium and contribute to the stability of microvessels (von Tell et al., 2006). Their position and abundance within the cerebral capillaries suggest that these cells play a major role in BBB integrity. The embryonic sources of pericytes include: a) neuroectoderm-derived neural crest cells, which give rise to pericytes of the forebrain, b) mesoderm-derived

mesenchymal stem cells, which give rise to pericytes in the midbrain, brain stem and spinal cord, and c) expansion by proliferation from the newly established pericytes pools (Winkler et al., 2011).

Pericytes and endothelial cells are connected to a shared basement membrane by several types of integrin molecule. N-cadherin is the key AJ protein between pericytes and endothelium (Perrot et al., 2020). Pairs of connexin 43 (CX43) hemichannels expressed respectively in pericytes and endothelium form gap junctions that allow transfer of molecules between pericytes and endothelial cells. CX43 is also abundant at astrocyte–endothelial cell and astrocyte-neuron interfaces (Winkler et al., 2011). The recruitment of pericytes to endothelium and the interaction between the two are essential factors for the formation, maturation and maintenance of the BBB (Armulik et al., 2011; Daneman et al., 2010; Su et al., 2019).

To date, it has been hypothesized that pericytes would deliver angiogenic factors that regulates permeability and vascular remodeling (Armulik et al., 2005; Sweeney et al., 2016), and that they would have contractile capabilities to allow them to regulate blood flow through the capillaries (Peppiatt et al., 2006). Moreover, pericytes play a major role in the differentiation of ECs during fetal life, even before the appearance of astrocytes (Armulik et al., 2011). Both neurodegeneration and impairments in memory and learning in pericyte-deficient mice have been demonstrated, it was suggested that pericytes control key neurovascular functions that are necessary for proper neuronal structure and function, and pericytes loss results in a progressive age-dependent vascular-mediated neurodegeneration (Bell et al., 2010). Pericytes also have a role in brain cholesterol homeostasis (Saint-Pol et al., 2012), beta amyloid (A β) clearance (Dalkara et al., 2011) as well as macrophage-like phagocytotic properties (Winkler et al., 2011).

1.5.6 Glial cells

Glial cells are the most abundant and widely distributed cells in the CNS; they interact with neurons and immune cells, as well as blood vessels. Virchow in 1846 was the first to find cells other than neurons in the brain (Baumann and Pham-dinh, 2002). There are two groups of glial cells in the CNS: the macroglia, including astrocytes, oligodendrocytes, and ependymal cells, and

the microglia (**Fig.9**). They interact among themselves and with neurons and endothelial cells around blood capillaries. Recently, neuron glia antigen-2 (NG2) glial cells, also referred to as oligodendrocyte precursor cells (OPCs), are considered the fourth type of glial cells in the CNS (Yang and Zhou, 2019). Glial cells form and maintain a highly controlled microenvironment that is essential for efficient neuron function within the CNS (Linnerbauer et al., 2020).

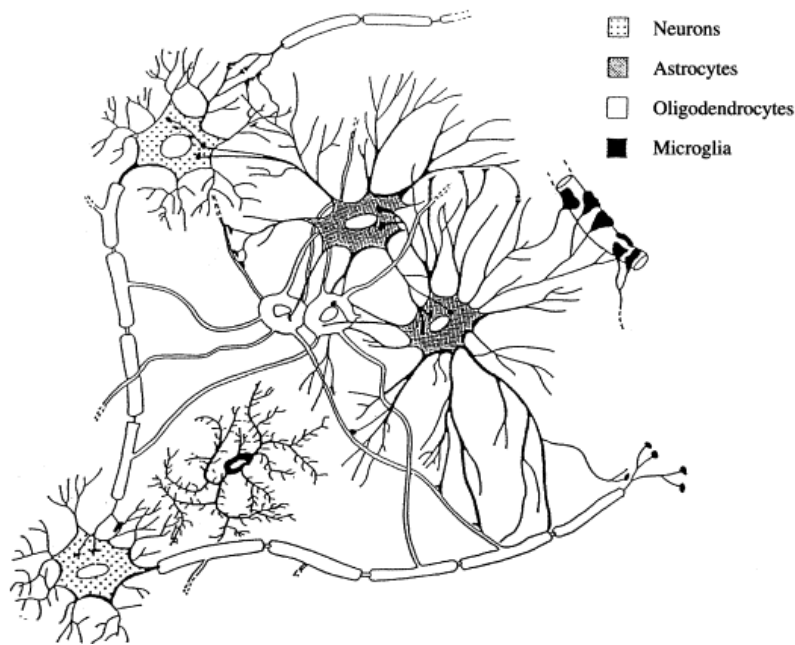


Figure 9. Schematic representation of the different types of glial cells in the CNS

Schematic representation of the different types of glial cells in the CNS and their interactions among themselves and with neurons and endothelial cells around blood capillaries. Adapted from (Baumann and Pham-dinh, 2002).

1.5.6.1 Astrocytes

Astrocytes are a class of neural cells originate from the neuroepithelium-derived radial glia within the subventricular zone (SVZ) that differentiates during embryogenesis (E17 in rodents) with the peak of astrogliogenesis occurring during late prenatal/early postnatal development (P1 to P10 in rodents) (Liddelow and Barres, 2015). They represent the most abundant cell type in the CNS and account for approximately 20–40% of all of the glial cells in the CNS (Ji et al., 2019) comprise at least 50% of the brain and spinal cord cells by number in humans and mice (Sofroniew and Vinters, 2010). Once astrocytes reach their final destination, axis

patterning cues guide the maturation of astrocytes into regionally-defined subgroups with a high degree of specialization of form and function, laying the foundation for their multifaceted roles in health and disease (Linnerbauer et al., 2020). Several morphologically distinguishable types of astrocytes exist, including protoplasmic (gray matter) and fibrous (white matter). Each subclass exhibits a degree of molecular heterogeneity that is only now beginning to be investigated (Sofroniew and Vinters, 2010; Verkhratsky and Nedergaard, 2018). Diversity of astrocyte functions and phenotypes in neural circuits is revised elsewhere (Khakh and Sofroniew, 2015).

Characteristic components of astrocytes include but not limited to type III intermediate filament proteins such as fibrillary acidic protein (GFAP) together with vimentin and desmin. The S100 calcium-binding protein β (S100 β) is considered as a calcium-binding protein of astroglia which acts as Ca^{2+} buffer as well as Ca^{2+} sensor, astrocytic glutamate transporters glutamate-aspartate transporter (GLAST) and glutamate transporter-1 (GLT-1), astroglia-specific enzyme converting ammonia and glutamate into glutamine (glutamine synthetase) as well as CX30, CX43, and aquaporin (AQP4) (Baumann and Pham-dinh, 2002) represent another way to characterization of astrocytes. For more information about the properties and functional relevance of the marker for astroglia and identification and morphology of astrocytes the reader can refer to (Baumann and Pham-dinh, 2002; Pekny and Nilsson, 2005; Verkhratsky and Nedergaard, 2018).

Historically, it has been supposed that astrocytes principally provide structural support and passive maintenance for neurons (Liddel and Barres, 2015). However, it is now evidenced that they also play an active role in many critical neural processes such as supporting neurons (Li et al., 2019) and formation and maintenance of the BBB (Dong and Benveniste, 2001).

Indeed, astrocytes occupy a strategic position between capillaries and neurons. They support neuronal functions in multiple ways (**Fig.10**) (Sofroniew and Vinters, 2010). Many interactions between astrocytes in the mature CNS, are regulated by gap junctions. Gap junctions formed by connexins build a pore through the cell membranes of two adjacent astrocytes, joining their cytosols and letting through certain sized molecules, including IP3 and potassium ions (Giaume et al., 2010). *In situ*, morphological studies have shown that astrocyte gap junctions are

localized between cell bodies, between processes and cell bodies, and between astrocytic end-feet that surround blood vessels. The main astrocytic gap junction proteins are Cx30 and Cx43, but others may be present (Abbott et al., 2006). CX43 is expressed around the twelfth day of gestation in rat, radial glial processes and CX30 is expressed in astrocytes during the third postnatal week and both of them are clearly co-expressed at junctional plaques of mature grey matter astrocytes (Giaume et al., 2010). Although many astrocytic processes are associated with synapses, structurally enwrapping and functionally interacting with dendritic spines and synaptic terminals by responding to neurotransmitters and by releasing gliotransmitters that regulate synaptic function (Perez-Alvarez et al., 2014). Other astrocytic processes are connected to each other by gap junctions formed by connexins, forming the glial network.

Astrocytes can modulate the extracellular levels of ions (K^+) and neurotransmitters which can affect the excitability of neurons. They can support neuronal metabolism by enhancing the release of energy substances such as lactate and pyruvate. Astrocytes can also take up glutamate and convert it to glutamine, which is then released into the extracellular space and taken up by neurons to resynthesize glutamate (Li et al., 2019). They also play an active role in other critical neural processes such as neurotrophic support, formation and maturation of inhibitory and excitatory synapses via a release of a range of molecules (for example: cholesterol, hevin, thrombospondins) that can control both pre- and postsynaptic function, synaptic pruning and inter-astrocytic communication (Liddelow and Barres, 2015). Astrocytes also secrete several growth factors such as brain-derived neurotrophic factor (BDNF), glial cell-derived neurotrophic factor (GDNF), and others that are fundamental for cell viability, oxidant protection, genetic expression and modulation of metabolic functions (Cabezas et al., 2016).

Those that form perivascular end-feet at the BBB have a special role in the homeostasis of the brain. In normal brain activity, neurons release neurotransmitters and K^+ and take up Na^+ , while glucose metabolism generates water. The neurotransmitters and ions are generally recycled, whereas water must be removed from the brain and excreted. Astrocytes contribute to ionic, amino acid, neurotransmitter and water homeostasis of the brain in several ways, and astrocytes that form perivascular end-feet at the BBB have a particular role in these processes

(Abbott et al., 2006). In addition astrocytes play an important role in the control of the blood flow by inducing release of prostanoids, arachidonic acid, and other molecules that signal vasodilation or vasoconstriction depending on metabolic context, coupling blood flow to neuronal energy demand (Liddelow and Barres, 2015). Astrocytes also influence the formation and maintenance of the BBB. Astrocyte ablation led to failure of BBB repair and edema (Bush et al., 1999). Cerebral edema, referring to the excess accumulation of fluid in the intracellular (cytotoxic edema) or extracellular (vasogenic edema) spaces in the brain (Jiang et al., 2018).

Astrocytes are pivotal in the formation of tight junctions of the ECs, the promotion of the enzyme system, glutamyl-transpeptidase 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 BBB (Cabezas et al., 2014; DeBault and Cancilla, 1980; Wong et al., 2013).

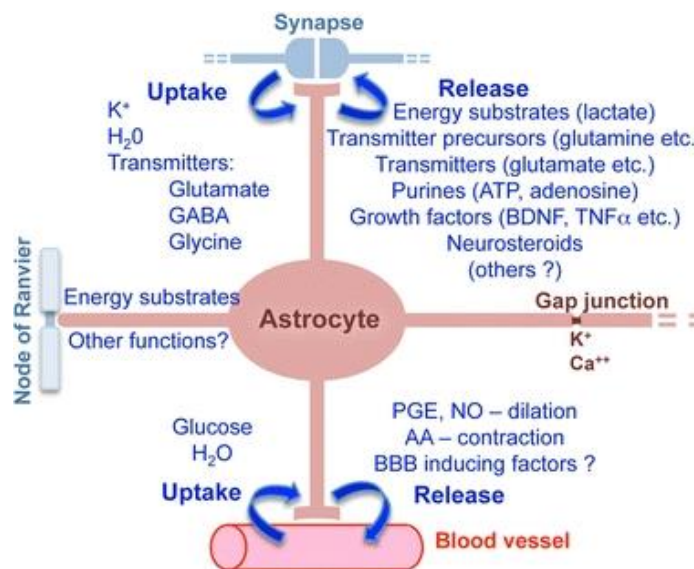


Figure 10. Schematic representation that summarize astrocyte functions in healthy CNS

Adapted from (Sofroniew and Vinters, 2010).

Moreover, Astrocytes are immune-competent cells able to detect danger signals, respond via secretion of cytokines and chemokines, and activate adaptive immune defense, thus the role of astrocytes as active players in cerebral innate immunity is important (Farina et al., 2007). Astrocyte reactivity can be triggered by a wide variety of molecules from diverse sources and

exhibited diverse functional responses and influenced many different cell types in a context-specific manner (Sofroniew, 2020). These effects provide benefit to the CNS, but as we will discuss, numerous evidences have demonstrated the negative outcomes of reactive-astrocyte responses as well.

1.5.6.2 Microglia

Microglia represents tissue-resident macrophages in the mammalian CNS, colonizing the CNS early in embryonic development. They are highly motile cells that interact with all cells in the CNS to mediate normal development, homeostasis, and general brain physiology. Comprising between 5% and 10% of the total number of CNS cells in humans and mice, they are integral to many physiological processes. They were described for the first time in 1919 by Del Rio-Hortega as a specific type of glial cell (see for review (Wolf et al., 2017)). There are some different hypotheses on the origin of microglia. The first hypothesis is based on a mesodermal origin (yolk sac mesoderm) of microglia during embryogenesis. The second hypothesis suggests that microglial precursors were subependymal cells (glioblasts, spongioblasts) which derived from the neuroectoderm. At last, the third hypothesis about the microglia origin postulates differentiation of microglyocytes from blood monocytes (Alekseeva et al., 2019). Summarizing varied contradictory data on the origin of microglial cells, it is possible to infer that their mesodermal descent now appears the most convincing. When circulation begins to form at the CNS, at a time when the BBB is still permissive, derived microglial cells can enter the CNS through the vascular network. Those microglial cells that enter the brain parenchyma during embryonic development, branch out then and remain quiescent (Davis et al., 1994). Microglial cells are activated during inflammatory reaction or immune challenge in the CNS and then differentiate into resident CNS macrophages (Johann and Beyer, 2013). Numerous studies have revealed that microglia interact with neurons during development and in the adult CNS. Homeostatic microglia functions that help to establish and to maintain the overall health of the nervous system include neuronal survival, removing dead cells from both the developing and the adult CNS through phagocytic machinery without initiating an inflammatory response (Nayak et al., 2014), pruning of supernumerary weak synapses, and formation of new synapses (Mosser et al., 2017). In addition, there is a bidirectional

communication between microglia and neuron in the healthy brain (**Fig.11**). The reciprocal neuron–microglia communication is mediated by numerous soluble factors, contact-dependent mechanisms (Pósfai et al., 2019; Szepesi et al., 2018), as well as extracellular vesicles (Frühbeis et al., 2013). Neurons can regulate microglial activation state by secreting factors (such as CD200 and fractalkine) that influence basal microglial properties as well as release neurotransmitters and purines that influence microglial behavior. Conversely, microglial cells also regulate neuronal activities and synapse development. It is shown that the microglia is also physically making contact with neuronal components (Eyo and Wu, 2013).

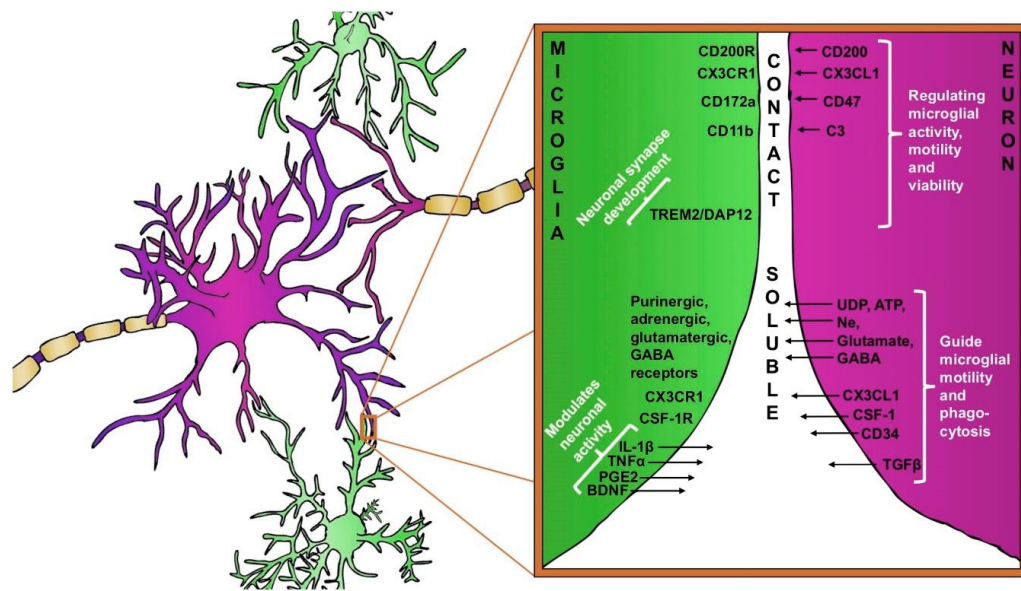


Figure 11. Bidirectional signaling between microglia and neuron

Bidirectional signaling between microglia (green) and neuron (purple). Neuron–microglia communication is mediated by receptor–ligand interactions as well as by various soluble factors. Microglia are equipped with a group of surface receptors, which trigger signals and regulate specific microglia function like phagocytosis, motility and viability. Many of the receptor ligands, such as CX3CR1, CD200R, and CD172a are released or expressed on the surface of neurons. Receptor–ligand interactions represent a classical contact dependent communication between microglia and neurons. Microglia and neurons reciprocally release soluble factors that can modulate cell functions and promote tissue homeostasis. Adapted from (Szepesi et al., 2018).

Several studies have documented that microglial cells interact with the neurovascular system. Both genetic and pharmacological approaches have suggested that microglia participates actively in the formation of new blood vessels or angiogenesis in both physiological condition and pathological contexts (Zhao et al., 2018). Lindberg *et al.* have introduced a reproducible model of inducing angiogenesis in the adult SVZ by extended intracerebroventricular infusion of epidermal

growth factor (EGF) (Lindberg et al., 2014). They have observed that during the growth of EGF-induced polyps, cell death increases and microglia gradually accumulates in the polyp core and adopts an amoeboid morphology. They observed that along with the peak of microglia accumulation, the first signs of angiogenesis appear and by increase of vascularization, cell death and microglia density decline, and polyp size increases substantially. Then, they conclude that microglia is associated closely to newly formed vessels (Lindberg et al., 2014).

A current study suggests that in the retina, microglial cells are involved in vascular development and angiogenesis (Bourghardt Peebo et al., 2011). Neuropilin-1 (NRP-1) is a transmembrane receptor that is critical for vascular development within the CNS. It binds and influences signaling of several key angiogenic factors, such as vascular endothelial growth factor (VEGF), semaphorin, platelet derived growth factor. NRP-1 is expressed by neurons and endothelial cells as well as a subpopulation of proangiogenic microglial cells that are thought to interact with endothelial tip cells to promote vascular anastomosis during brain vascularization. It has already shown that NRP-1-expressing microglia are major contributors to pathologic retinal angiogenesis (Dejda et al., 2014). The involvement of microglia-resident NRP-1 in developmental retinal vascularization has also been shown (Dejda et al., 2016). The microglia interact also with the NVU and this interaction is bidirectional and dynamic, involving several mechanisms and mediators. Microglia exert dual detrimental (BBB damage and inflammation) and protective (BBB repair and angiogenesis) properties on the BBB (Thurgur and Pinteaux, 2019).

1.5.7 Neuron

Active neurons send various signals to glial cells and blood vessels in order to regulate the CBF and to obtain sufficient O₂ and substrates. As already mentioned in anatomical level, astrocytes surround most of the arteriolar and capillary abluminal surface with their end-feet and are uniquely positioned between synapses and vessels. This specific arrangement led to the concept of a neuron-astrocyte-vasculature tripartite (**Fig.12**). Astrocytes can integrate neurotransmitters signals from thousands of synapses and relay this information to the arterioles (Duchemin et al., 2012).

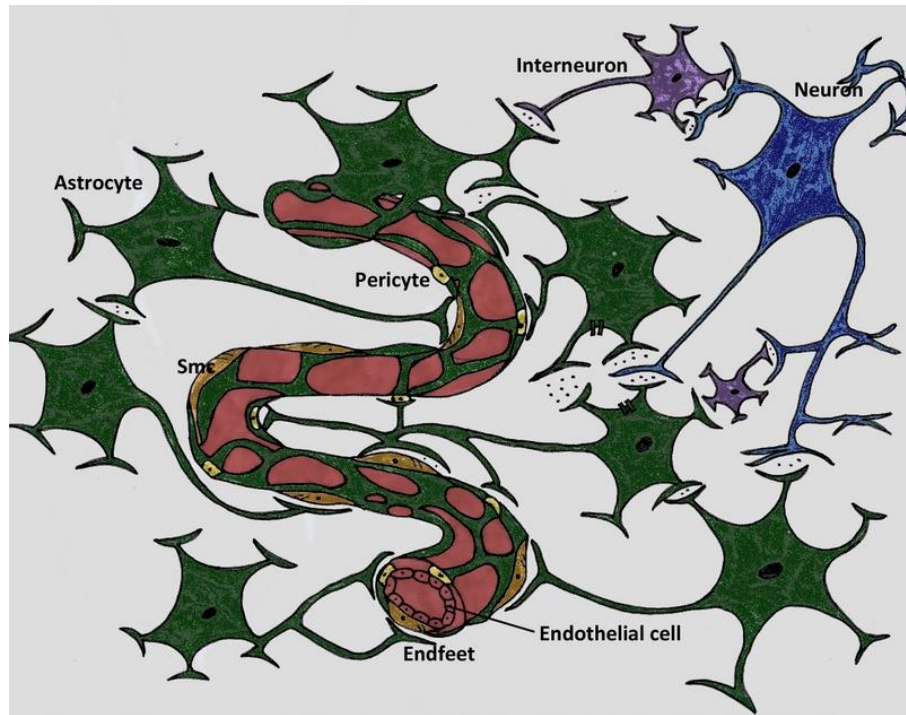


Figure 12. neuron-astrocyte-vasculature tripartite

The neurone (blue) establishes synapses with interneurons (purple) and with astrocytes (green), sending information through neurotransmitters on their oxygen requirements and commands contraction or vascular relaxation. Astrocytes, through calcium waves, transmit the order to the vessels. The point of contact between astrocytes and the vasculature is the endfeet, which can be in touch directly with endothelial cells (red), pericytes (yellow). Adapted from (Muio et al., 2014).

Due to the dynamic nature of neural activity and the considerable metabolic needs of the nervous tissue, the communication between neurons and the vasculature is pivotal in the regulation of blood flow and supply (Hawkins and Davis, 2005). Indeed, neurons communicate with the vessels either directly through the astrocytes, or via an interneuron, thus influencing the behavior of capillary endothelial barrier (Muio et al., 2014). There is evidence for direct innervation of both brain microvessel ECs and associated astrocyte processes via distinct connections with noradrenergic, serotonergic, cholinergic and GABAergic neurons and have influence on the behavior of capillary endothelial barrier resulting in influencing the vascular tone and consequently the blood supply to the area surrounding it (Muio et al., 2014; Persidsky et al., 2006). It was shown that NVU astrocytes are able to detect glutamate (Zonta et al., 2003) and GABA (Muio et al., 2014) neuronal levels and convert those signals into vasomotor commands. It is important to note that multiple factors such as direct innervation of vessels and multiple

signaling pathways contribute to functional CBF. Various experimental approaches support a role of neuronal nitric oxide (NO) in the maintenance of resting CBF as well as in the vascular response to neuronal activity (Duchemin et al., 2012). 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 pathways (Ridnour et al., 2007). 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) (Ridnour et al., 2007). It has been shown that axons, dendrites or soma of NOS interneurons contact the basement membrane of blood vessels or perivascular astrocytes in all segments of the cortical microcirculation.

A great study also suggested that neuron-to-astrocyte signaling is a key mechanism in functional CBF (Zonta et al., 2003). In fact, during high synaptic activity, glutamate released from axonal terminals diffuses to astrocyte membranes located at the border of the synaptic cleft. The ensuing activation of metabotropic glutamate receptors ((mGluRs are known to mediate the Ca^{2+} increase of astrocytes in response to synaptic released glutamate) triggers Ca^{2+} oscillations on astrocyte processes that spread to the end-feet that are in contact with arterioles and this Ca^{2+} elevation regulates the release of a vasoactive agent, most likely a cyclooxygenase product, from astrocytes (presumably from their end-feet) that leads to blood flow increase (Zonta et al., 2003). Altogether, when necessary, neurons communicate with the vessels via the intermediate of astrocytes, then astrocytes convert those signals into vasomotor commands.

1.6 Blood-brain barrier transport systems

TJ and AJ both prevent free paracellular exchanges of solutes, and lack of fenestration and extremely low rates of transcytosis greatly limit transcellular diffusion (Ballabh et al., 2004). The latter two features arise from several changes to ECs morphology and biochemistry that occur during embryonic development, thus allowing the transition from a permeable to a continuous vessel (Obermeier et al., 2013). As part of their normal function, ECs not only impede the influx of intravascular substances from blood to brain, but also promote transport of substances from

blood to brain or from brain to blood through several transport systems (Deeken and Löscher, 2007). Cells, such as immune cells, may cross the BBB through or adjacent to the tight junctions. Solutes may passively diffuse through the cell membrane. Active efflux carriers may pump some of these passively penetrating solutes out of the endothelial cell. Carrier-mediated influx (passive or secondarily active) can transport essential polar molecules into the CNS. Transcytosis mediated by either receptor-mediated transcytosis (RMT) which transports macromolecules across the endothelium or adsorptive-mediated transcytosis (AMT) is induced non-specifically by positively charged macromolecules and can result in passage across the BBB. TJ modulation may occur, affecting the permeability of the paracellular aqueous diffusional pathway (Serlin et al., 2015) (Fig. 13).

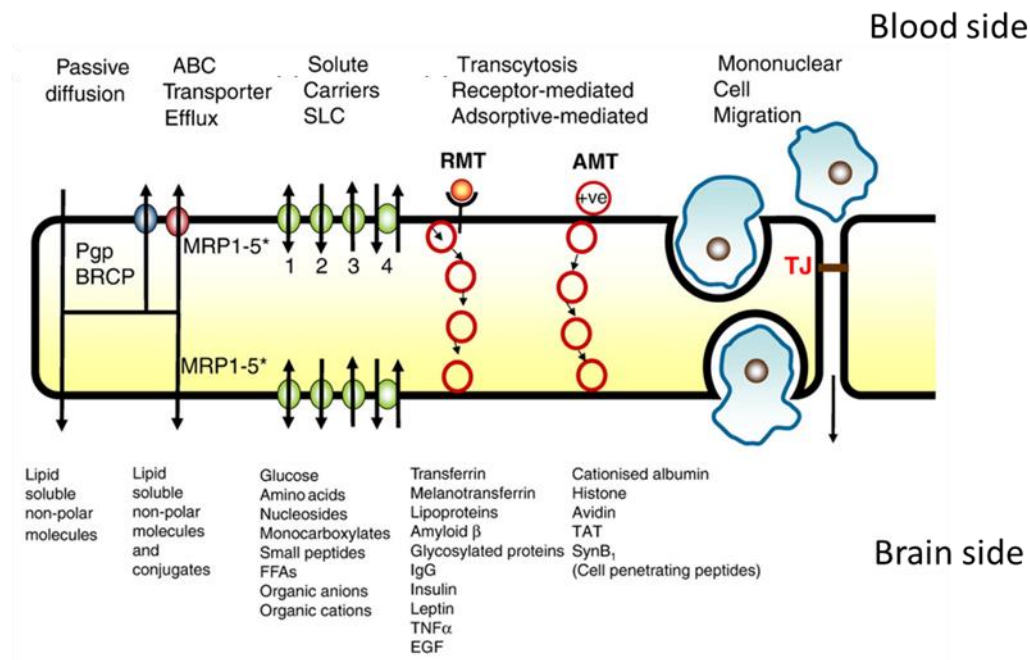


Figure 13. Routes of transport across the BBB

Adapted from (Abbott et al., 2010)

1.6.1 Passive diffusion

Simple diffusion, a type of passive diffusion, is the process by which solutes are moved along a concentration gradient in a solution or across a membrane. O_2 and CO_2 and small lipophilic molecules such as ethanol cross the BBB by simple diffusion. There is a general correlation

between the rate at which a solute enters the CNS and its lipid solubility, thus a higher lipid solubility and several other physicochemical factors favor passive process (Sweeney et al., 2018).

1.6.2 ATP-binding cassette active efflux transporters

ATP-binding cassette (ABC) transporters are ATP-driven efflux pumps that are expressed by the capillary ECs of the BBB. Together with other transporters, they form a dynamic barrier at the blood-brain interface by extruding various lipophilic compounds from the brain back into the blood circulation (Schinkel and Jonker, 2003). ABC active efflux transporters limit also entry of drugs, xenobiotics, and drug conjugates. Efflux transporters, including multi drug resistant-1 (MDR1) (also called P-glycoprotein, P-gp), breast cancer resistance protein (BCRP), and multidrug resistance-associated proteins (MRPs), use the hydrolysis of ATP to transport their substrates against their concentration gradient (Li and Sahi, 2016). Many of these transporters are localized to the luminal surface and transport a wide array of substrates into the blood compartment. This wide substrate diversity allows these transporters to provide a barrier to many small lipophilic molecules, which would otherwise passively diffuse through the endothelial membrane (Daneman and Prat, 2015). Active efflux carriers may intercept some of these passively penetrating solutes and pump them out of the endothelial cell either as they diffuse through the cell membrane or from the cytoplasm (Serlin et al., 2015). Notably, efflux transporters (P-gp, BCRP and the OAT3) are also responsible for the transport of several classes of pharmaceutical drugs (Choudhuri and Klaassen, 2006; Liu and Liu, 2019). Besides, the efflux transporters also present a major obstacle in central distribution of many therapeutic agents that are beneficial to treat CNS diseases. A numerous of lipid-soluble molecules such as some therapeutic drugs are the substrates for the efflux transporters. Some efflux transporters limit brain uptake of these agents in order to protect the brain from potentially harmful exogenous substances (Choudhuri and Klaassen, 2006; Löscher and Potschka, 2005). Additionally, the unique cellular phenotype in cerebrovascular endothelial cells has been represented by the enrichment of genes coding transporters in transcriptome, which accounts for more than 10% of gene expressions in this cell type (Yamazaki and Kanekiyo, 2017).

1.6.3 Solute carrier-mediated transport

The solute carrier-mediated transport (CMT), facilitated diffusion of metabolites, nutrients, vitamins, nucleotides and carbohydrates, amino acids, monocarboxylates, hormones, fatty acids, organic anions and cations, amines and choline with precise substrate specificity and directionality, according to substrate specificity and concentration gradient. Since the gradient in concentration across the BBB is the major driving factor for CMT, the pathway is likely affected by the size, affinity, and physicochemical properties of each specific molecule (De Bock et al., 2016). Carrier mediated influx via solute carriers may be passive or primarily or secondarily active and can transport many essential polar molecules such as glucose, amino acids and nucleosides into the CNS (Girardin, 2006). The solute carriers may be bi-directional, the direction of net transport being determined by the substrate concentration gradient, unidirectional either into or out of the cell, or involve an exchange of one substrate for another or be driven by an ion gradient. In this last case the direction of transport is also reversible depending on electrochemical gradients (Abbott et al., 2010).

1.6.4 Transcytosis

Transcytosis is the transcellular transport of molecules using vesicles. Macromolecules are first endocytosed or internalized by vesicles on one side of the cell, trafficked in vesicles and then exocytosed or released on the other side of the cell. Transcytosis in CNS endothelial cells can be divided into two categories: Receptor-mediated transcytosis (RMT) (in the form of caveolae or clathrin-mediated mechanisms) and the nonselective adsorptive-mediated transcytosis (AMT) (Ayloo and Gu, 2019).

RMT transport system is implicated in the transfer of peptides and proteins including transferrin, insulin, leptin, arginine vasopressin, amyloid- β , glycosylated proteins, and apolipoproteins E (APOE) and J (APOJ). AMT appears to be induced in a non-specific manner by positively charged macromolecules and can also transport across the endothelium (Pulgar, 2019).

The two major endocytic RTM pathways at the BBB, are clathrin-coated-vesicles- and caveolae-mediated transport. Caveolae or “small caves” were originally identified as 50–100 nm

flask-shaped, non-clathrin-coated vesicles with specific coat and adapter protein components, i.e., caveolins (Cav) and the more recently discovered cavins (Deng et al., 2011). To date, Cav-1, Cav-2 and Cav-3 caveolin isoforms have been identified in murine, bovine, and human cerebral endothelial and astroglial cells (Ikezu et al., 1998). A large number of signaling molecules that regulate vascular ECs localize to lipid rafts/caveolae (**fig.14A**). These include receptors, e.g., receptor tyrosine kinase (RTK), G-protein-coupled receptors (GPCRs), transforming growth factor-beta (TGF- β) type I and II receptors, certain steroid receptors, low molecular weight and heterotrimeric G-proteins, and “downstream” enzymes and components including endothelial NOS. Caveolae contain key proteins known to mediate different aspects of vesicle formation, docking, and fusion (Schnitzer et al., 1995). Cavin-1, cavin-2, cavin-3 and cavin-4 may be important in regulating caveolin expression and caveolar morphology (Sowa, 2012). It has been shown that the presence of cavin on the inner surface of caveolae stabilizes these structures, probably through interaction with the cytoskeleton, and cavin therefore plays an important role in caveolae formation and organization (Liu and Pilch, 2008). The adapter proteins of caveolae, cavins provide the scaffold, determine shape, and regulate internalization/budding of caveolae (**fig.14B**).

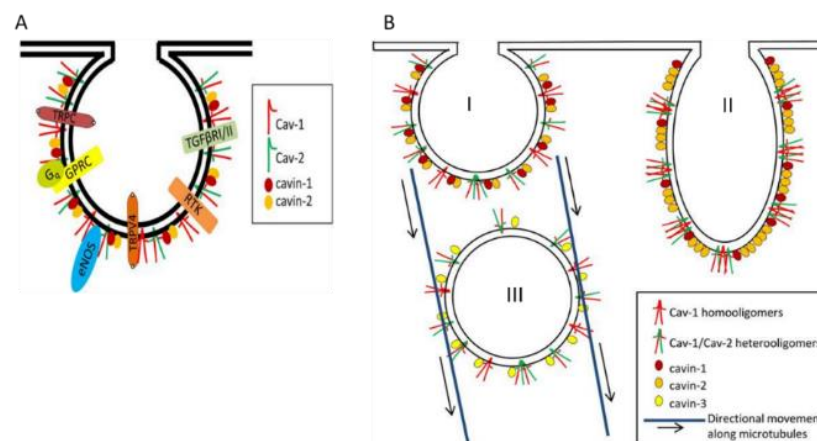


Figure 14. Signaling proteins localized in endothelial cell caveolae and The complex role of caveolar coat in caveolae formation

A) Several signaling molecules were localized in endothelial caveolae and interact with Cav-1 including endothelial NOS, RTK (e.g., VEGF Receptor 2), GPCR (e.g., Bradykinin Receptor 2, Endothelin Receptor, Muscarinic Receptor), heterotrimeric G protein subunits (e.g., Gq), TGF-beta Receptors I and II, calcium channels (e.g., TRPC1 and 4, TRPV4). B) The complex role of caveolar coat and adapter proteins in caveolae formation (I), deformation/elongation (II), and internalization (III). Adapted from (Sowa, 2012).

Cavin-1 is a soluble protein which is recruited to caveolae as a scaffold stabilizing the caveolae unit. Cavin-2 and cavin-1 interact with each other and cavin-2 promotes recruitment of cavin-1 to caveolae. Cavin-2 is thought to be a necessary component for inducing membrane-curvature of caveolae. Cavin-3 associates with Cav-1 upon caveolae internalization to form vesicles and its absence markedly reduces intracellular Cav-1 traffic along microtubules (Sowa, 2012). A great review is available discussing how various EC signaling molecules and their functions are regulated by caveolae and their specific coat and adapter protein components such as caveolins and cavins (Sowa, 2012).

1.6.5 Other transport systems

Ion concentrations are regulated by the abluminal sodium pump (Na^+ , K^+ ATPase), the luminal sodium-hydrogen exchanger, chloride-bicarbonate exchanger, luminal sodium-potassium-chloride cotransporter, and sodium-calcium exchanger. Water is transported via aquaporin (AQP) receptors: AQP1 on endothelial cells and AQP4 on astrocytic end-feet (Sweeney et al., 2018). Additionally, leukocytes cross the BBB either by a process of diapedesis through the endothelial cells (penetrating close to the tight junctional regions), or via modified tight junctions. The JAMs and the cell surface protein CD99 may interact with the leukocytes to initiate diapedesis. TJs modulation can result from signals from cells associated with the brain endothelium or be induced pharmacologically; molecular re-arrangement of the proteins of the tight junctions results in complete or partial opening of the paracellular aqueous diffusional pathway (Abbott et al., 2010).

1.7 Concept of the neurovascular unit

The neurovascular unit (NVU) defined as a group of closely related components, includes neurons, glial cells (astrocytes and microglia), vascular wall cells (endothelial cells, pericytes), smooth muscle cells and the basement membrane (Wang et al., 2021), and all are involved in regulating cerebral blood flow and BBB function (Muoio et al., 2014). The concept of the NVU emerged from the first meeting in July 2001 of the National Institute of Neurological Disorders and Stroke (July 2001) reviewing stroke research progress to emphasize the intimate relationship

between the brain and its vessels (Iadecola, 2017). This is a relatively recent concept in neuroscience that broadly describes the relationship between brain cells and cerebral blood vessels. Since then, the NVU has attracted the interest of the neuroscience community, resulting in considerable advances in the field.

1.8 Neurovascular unit dysfunction in CNS disorders

BBB breakdown (due to disruption of the TJs or altered transport of molecules between blood to brain and brain to blood), aberrant angiogenesis, vessel regression, brain hypoperfusion and inflammatory responses, may initiate and/or contribute to a “vicious circle” of the disease process, resulting in a progressive synaptic and neuronal dysfunction and an increase in disorders such as Alzheimer's disease (AD), Parkinson's disease (PD), Huntington disease, multiple sclerosis (MS), and others (Zlokovic, 2008). Disruption of the BBB is also associated with peripheral diseases such as diabetes (Prasad et al., 2014) and non-neural inflammatory-related pathologies (Liu et al., 2017b) as well as acute neurological disorders such as stroke (Khatri et al., 2012), traumatic brain injury (Habgood et al., 2007), spinal cord injury (Kumar et al., 2018) and epilepsy (Rempe et al., 2018) or other chronic neurodegenerative disorders such as chronic traumatic encephalopathy and schizophrenia (Farrell et al., 2019) and HIV-1-associated dementia (András et al., 2017). Altogether, impairments of the NVU widely occur in numerous CNS disorders which are characterized by an increase of BBB permeability and the entrance of proinflammatory and neurotoxic blood-derived factors into the brain parenchyma, thus may providing a starting point of neurodegenerative process and complications of many brain disease and injuries (Liebner et al., 2018; Sweeney et al., 2018).

It was suggested that the BBB dysfunction in MS, epilepsy, and stroke is a central element of the pathology, whereas in others, the incidence and extent of breakdown are more controversial. Moreover, the mechanisms underlying the BBB dysfunction and its role in the onset and progression of disease or recovery are not fully understood.

1.8.1 Alzheimer's disease

Alzheimer's disease (AD) refers to a particular cognitive and functional decline associated with aging together with a particular neuropathology. The pathophysiology of AD is still subject to some controversy. The prevailing hypothesis places the accumulation of A β at the center of the process and is referred to as the "amyloid-cascade hypothesis" (Soria Lopez et al., 2019). AD is characterized by amnesic-type memory impairment, language deterioration, and visuospatial deficits correlate with neuronal degeneration, loss of synapses, neurofibrillary tangles, gliosis and A β accumulation in senile plaques and cerebrovasculature mainly in the hippocampus and neocortex (Zenaro et al., 2017). Numerous studies suggest that neurovascular dysfunction contributes to the onset and progression of AD and propose a link between cerebrovascular changes and neurodegeneration. A β deposition in the vasculature leads to pro-inflammatory and cytotoxic events that contribute to the greater BBB permeability in the AD brain. BBB impairment, due to disruption of the TJs (Marco and Skaper, 2006) and AJs (Muradashvili et al., 2014), altered transport of molecules between blood to brain (Kalaria and Harik, 1989) and brain to blood (Zlokovic et al., 2010), cerebrovascular inflammation (Tripathy et al., 2013), oxidative stress and ROS generation (Feng and Wang, 2012), basement membrane alteration (Zipser et al., 2007), pericyte dysfunction (Zenaro et al., 2017) and detachment (Sengillo et al., 2013) and leukocyte trafficking across the BBB (Zenaro et al., 2017), may initiate and/or contribute to disease process, resulting in progressive synaptic and neuronal dysfunction and loss. The evidence of BBB disruption derived from analyses of post-mortem tissues from patients with AD and other neurodegenerative disorders revealed brain capillary leakages, degeneration of BBB-associated cells (including pericytes and endothelial cells), brain infiltration of circulating leukocytes and red blood cells, aberrant angiogenesis and molecular changes (Sweeney et al., 2018).

1.8.2 Parkinson's disease

Parkinson's disease (PD) is a common and complex neurological disorder with early prominent death of dopaminergic neurons in the substantia nigra pars compacta (SNpc). The resultant dopamine deficiency leads to movement disorders (Kalia and Lang, 2015). It has been suggested that vascular risk factors accelerate the onset and severity of motor and cognitive

impairments during early stages of PD, and that cerebrovascular dysfunction can influence development and progression of PD. Vascular dysfunction occurs throughout the basal ganglia of PD patients, consisting of BBB breakdown and dysfunction and diminished active efflux of xenobiotics and other potential toxins. Aberrant angiogenesis with increased number of endothelial cells, decreased TJ and AJ proteins, and capillary basement membrane changes have been shown both in humans with PD and animal models (Sweeney et al., 2019). BBB breakdown can also lead to neuroinflammation, neurotoxic accumulates of fibrinogen, thrombin, and plasminogen, and red blood cell extravasation, release of hemoglobin and iron (Fe^{2+}) causing ROS induction, which all can injure dopaminergic neurons (Stephenson et al., 2018; Sweeney et al., 2019).

1.8.3 Stroke

According to the World Health Organization (WHO), stroke is the 'incoming epidemic of the 21st century'. A stroke is a medical condition in which poor blood flow to the brain causes cell death. There are two main types of stroke: ischemic, due to lack of blood flow, and hemorrhagic, due to bleeding. Both of them pose a significant burden on global health (Amarenco et al., 2009). BBB dysfunction is a prominent pathological characteristic of both ischemic and hemorrhagic stroke. (Jiang et al., 2018). It is now evident that neuroinflammation contributes to BBB damage in ischemic stroke. In the early phase of ischemic stroke, inflammation may trigger ischemic injury, whereas inflammatory responses may be neuroprotective at late stages by promoting recovery and facilitating neurogenesis, angiogenesis, and neuroplasticity (Yang et al., 2019). In addition to glial activation and increase of inflammatory signal (Yang et al., 2019), alterations of endothelial junctional proteins through protein degradation (Abdullahi et al., 2018) or translocation (Liu et al., 2012), cytoskeletal rearrangement and modulation of ECM components (Edwards and Bix, 2019), alteration of transcellular diffusion mechanisms (Jiang et al., 2018), astrocyte swelling due to increased uptake of glutamate and lactate (Kimelberg, 2005), pericytes constriction and their migration or detachment from the microvessel walls (Dalkara et al., 2019), have been documented. Following stroke, the BBB leakage as a consequence of above mentioned-

modification of the BBB structure and function result in cerebral edema, non-homeostasis and injury progression.

For more information regarding neuroimaging studies in the living human brain, post-mortem tissue and biomarker studies demonstrating BBB breakdown in mentioned disorders and pathogenic mechanisms by which BBB breakdown leads to neurodegeneration see reference (Ransohoff, 2016; Sweeney et al., 2019; Zlokovic, 2008).

1.9 Determinants of neurovascular unit alteration

The integrity of NVU depends on the proper functioning of each element of this complex structure. Alteration of only one of the components of the NVU complex leads to BBB dysfunction (Hawkins, 2005). Factors that can disrupt the BBB are varied, ranging from pathogens and CNS diseases to physiological ageing. Generally, neuroinflammation, oxidative stress and MMPs activation are known as the major cause for NVU breakdown and BBB dysfunction, in turn resulting in non- hemostasis and contributing to the progression of neurological diseases (Hawkins, 2005; Reinhold and Rittner, 2017). Along with the fact that aging is commonly associated with progressive deterioration in CNS function, it is one of the important risk factors for the damage of NVU. Moreover, hypogonadism represents an emerging topic of research in the context of NVU dysfunction.

1.9.1 Neuroinflammation

Historically, the CNS was considered an immuno-privileged organ, lacking a lymphatic system and shielded from the peripheral circulation by the BBB. However, it is now clear that the BBB has the ability to respond to soluble factors and plasma proteins, and to communicate with peripheral immune system cells thus establishing neuro-immune system interactions. Therefore, the brain can no longer be viewed as an immuno-privileged organ and NVU dysfunction should be considered in a broader context, including peripheral immune cells and circulating soluble molecules that mediate immune responses (Zenaro et al., 2017). Inflammation is an adaptive response that is triggered by noxious stimuli and conditions, such as infection, tissue injury and

issue stress. The inflammatory response has a different physiological purpose such as restrain infection, tissue-repair response, and adaption to stress. Based on the context, course, and duration of the inflammatory responses, inflammation can become persistent then lead to pathological consequences such as autoimmunity, inflammatory tissue damage and sepsis, fibrosis, metaplasia and/or tumor growth and shift in homeostatic set points (Mukhara et al., 2020; Sherwood and Toliver-Kinsky, 2004).

The innate immune response of the neural tissue that is centralized within the brain and spinal cord, is generally referred to as neuroinflammation (DiSabato et al., 2016; Medzhitov, 2008). Neuroinflammation is a complex and well-orchestrated process by various groups of glial cells in CNS and peripheral immune cells. The cross-talks between various groups of glial cells in CNS neuroinflammation represent an extremely complex and dynamic process which resembles a well-orchestrated symphony. However, the understanding of how glial cells interact with each other to shape the distinctive immune responses of the CNS remains limited (Linnerbauer et al., 2020). However, the cellular and molecular mechanisms of how glial cells work in neuroinflammation remain poorly understood for several reasons. First, neuroinflammation is not a simple response; it is highly variable following different inflammatory cues, such as infection, mechanical damage, toxic metabolites, and autoimmunity. Glial cells are known to play distinct roles under different scenarios of neuroinflammation. Second, similar to immune cells, glial cells play “double-faced” roles in the processes of neuroinflammation, depending on the course of disease and certain environments of inflammation. Third, the outcomes of neuroinflammation are the result of the joint actions of multiple glial cell types and peripheral immune cells (Yang and Zhou, 2019). The hallmarks of neuroinflammation are the presence of activated glial cells and increased production of the inflammatory mediators.

1.9.1.1 Activated astrocytes

Astrocytes have a capacity to react mainly in relation to an injury in the CNS and constitute reactive astrocytes (astrogliosis). This reaction is characterized by the change in morphology, transcriptome levels and number of astrocytes (Baumann and Pham-dinh, 2002). Indeed the ratio of astrocytes to neurons increases with the increasing complexity of the CNS (Pekny and Pekna,

2004). These changes can exert both beneficial and detrimental effects in a context-dependent manner. It is important to note that neuroinflammation can lead to either astrocyte loss or astrogliosis. In contrast to neuroinflammation in neurodegenerative disorders which is accompanied with astroglial proliferation, several inflammation conditions such as Rasmussen's encephalitis, psychiatric disorders such as depressive disorder, bipolar disorder and schizophrenia as well as neuromyelitis optica, neuroinflammation is commonly associated with loss of astrocytes. Loss of astrocytes can be caused by cytotoxic T cells, antibodies or cytokines (Hostenbach et al., 2014). Thus dysfunction of astrocytes or the process of reactive astrogliosis is emerging as an important potential source of mechanisms that might contribute to, or play primary roles in, a host of CNS disorders either via loss of normal or gain of abnormal astrocyte activities (Hamby and Sofroniew, 2010). The cellular and molecular mechanisms leading to astrogliosis are not completely understood, but when damage occurs in the CNS, many different types of intercellular signaling molecules are able to trigger reactive astrogliosis or to regulate specific aspects of reactive astrogliosis including (1) large polypeptide growth factors and cytokines such as Interleukin- (IL) IL-6, IL-1, IL-10, leukemia inhibitory factor (LIF), TNF α , interferon gamma (IFN γ), Transforming growth factor beta (TGF β), etc., (2) mediators of innate immunity such as lipopolysaccharide (LPS) and other Toll-like receptor ligands, (3) neurotransmitters such as glutamate and noradrenalin, (4) purines such as ATP, (5) reactive oxygen species (ROS) including nitric oxide (NO), (6) hypoxia and glucose deprivation, (7) products associated with neurodegeneration such as β -amyloid, (8) molecules associated with systemic metabolic toxicity such as NH $_4^+$, and (9) regulators of cell proliferation such endothelin-1, as reviewed in detail elsewhere (Sofroniew and Vinters, 2010). Such molecular mediators may originate from all cell types in CNS tissue, including neurons, microglia, oligodendrocyte lineage cells, pericytes, endothelium, and astrocytes, in response to all forms of CNS insults: they play an important role as triggers and modulators of astrogliosis (Hostenbach et al., 2014; Sofroniew and Vinters, 2010).

Upregulation of intermediate filament (IF) proteins, in particular the glial fibrillary acidic protein (GFAP) by reactive astrocytes is perhaps the best known hallmark of reactive astrocytes (Pekny and Nilsson, 2005) (**fig.15**). Not all astrocytes in healthy CNS tissue express detectable level of GFAP. In contrast, a mild, moderate or severe astrogliosis reveals variable changes in molecular

expression, functional activity and cellular hypertrophy. At this stage, most of the astrocytes are GFAP-positive (Sofroniew and Vinters, 2010).

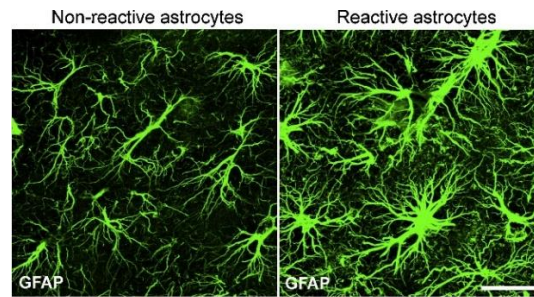


Figure 15. representative Images of astrocytes responding to injury.

In response to injury, cellular processes of reactive astrocytes show signs of hypertrophy. Immunodetection of intermediate filament protein GFAP (green) reveals astrocytes in an unchallenged mouse hippocampus (left) and activated astrocytes in the mouse hippocampus, 4 days after entorhinal cortex lesion, the latter with clear signs of hypertrophy of astrocyte processes (right). Adapted from (Pekny et al., 2014).

Recent studies have demonstrated that neuroinflammation can elicit two different types of reactive astrocytes, termed A1s and A2s (Liddelow and Barres, 2017; Liddelow et al., 2017). The authors identified A1 neurotoxic reactive astrocyte formation as a fundamental pathological response of the CNS to LPS-induced neuroinflammation, acute CNS injury, and most or all neurodegenerative diseases. In contrast, A2 reactive astrocytes, which are induced by ischemia strongly promote neuronal survival and tissue repair. For example (Hayakawa et al., 2014) provided proof that A2 reactive astrocytes promote adhesive interactions between brain endothelium and endothelial progenitor cells which is pivotal for neurovascular repair after stroke and neurodegeneration (Hayakawa et al., 2014). However, in the case of astrocyte reactivity in different neurodegenerative diseases, these issues remain a high level of conflict and controversy (Li et al., 2019). It has been suggested that TGF β , IFN γ , IL-6 β , estrogen, STAT3, and BDNF support the protective phenotype of the astrocyte, whereas IL-17, sphingolipids, NF κ B (nuclear factor-kappa B), chemokines and vascular endothelial growth factor (VEGF) trigger damaging pathways (Colombo and Farina, 2016).

We now know that the astrocyte response machinery includes phagocytosis of synapses, changes in the secretion of neurotrophins, clearance of debris and dead cells, and repair of the BBB, as well as formation of a scar to enclose the necrotic lesion of such injuries or infection.

These effects provide benefit to the CNS, but as we will discuss, evidence also points to negative outcomes for responses of reactive astrocytes (Liddelow and Barres, 2017). Astroglia–endothelial signaling in infection or inflammation, stroke or trauma, lead to opening of the BBB and disturbance of brain function by multiple pathways. Astrocytic inflammatory mediators alter BBB permeability and TJ protein expression. For example, bradykinin, a peptidergic mediator of inflammation, is produced during cerebral trauma and ischemia. Bradykinin can act on endothelial and astroglial bradykinin B2 receptors, leading to an increase in the concentration of intracellular Ca^{2+} . It induces cytokine production in astrocytes through activation of the NF- κ B (Schwaninger et al., 2002). The transcription factor NF- κ B controls multiple aspects of astrocyte pro-inflammatory responses. NF- κ B signaling is also triggered/boosted by cytokines released by microglia and other cells in the inflamed CNS (Linnerbauer et al., 2020). Other central and peripheral substances are released during the inflammation condition, (substance P, 5-hydroxytryptamine and histamine) which affect astrocytes and can lead to the formation of cytokines such as prostaglandins (PGs), with effects on vascular tone and endothelial permeability (Abbott et al., 2006).

Moreover, in the pathological condition, AQP4 is upregulated in the astroglial end-feet, leading to astroglial swelling which results in cerebral edema (Abbott et al., 2006). Indeed, a crucial role of AQP4 is to rapidly neutralize osmotic gradients generated by ionic transporters. The formation of cerebral edema results in an increase in tissue water content and brain swelling which, if unchecked, can lead to elevated intracranial pressure, reduced cerebral blood flow, and ultimately cerebral herniation and death (Zador et al., 2009).

Moreover, an *in vitro* BBB model which has investigated the interaction between endothelial cells and astrocytes, points out the importance of astrocytes' mediation in inducing endothelial sensitivity to $\text{A}\beta$. The presence of astrocytes is essential to induce endothelial response to $\text{A}\beta$, resulting in increased barrier permeability and TJ disruption. In fact, astrocytes contribute to $\text{A}\beta$ -induced BBB damage through at least in part activation of endothelial MMP-9 (Spampinato et al., 2017).

1.9.1.2 *Microglia activation*

Microglia are remarkably plastic and capable of responding to a vast array of challenges. Upon detection of specific factors generated by parenchymal injury, degeneration, or infection, microglia undergo morphological transformations and respond rapidly through induction of genetic programs designed to overcome and repair CNS insults. In the normal “resting” state, microglia demonstrates a small cell body and high ramified shape with extended pseudopodia and uppressed genomic activity (Wood, 2002).

Transformations of microglial morphology, phenotype, and function are observed during almost all neuropathological conditions including degenerative diseases, infection, stroke, tumors, brain injury (Nayak et al., 2014). The activated and the phagocytic stages of microglia which is accompanied by a genomic upregulation, lead to the production of a large number of potentially neurotoxic mediators but crucial to the “housekeeping” activities of microglia. However, it now appears that in a number of clinical conditions and in a number of preclinical models, microglia remains in an activated state for extended periods and may contribute to neuronal lysis by direct cytotoxic actions of some microglial mediators (Wood, 2002). Microglia expresses a specific marker, the Iba-1 protein (ionized calcium binding adapter molecule 1), which is upregulated during microglial activation (Ito et al., 1998; Sasaki et al., 2001).

Microglial mediators include cytokines, proteases, prostaglandins, acute-phase proteins, amyloid precursor protein, transmembrane proteins involved in cytotoxicity or astrogliosis, nitric oxide and, free radicals. Within the CNS, microglia are the major cellular source for the proinflammatory cytokines such as IL-1 α , IL-1 β , and TNF α , as well as inflammatory and chemokinetic cytokines such as macrophage inflammatory protein-1 α (MIP-1 α), IL5, and IL8. The proinflammatory cytokine and chemokinetic cytokine production result in local inflammatory responses and neuronal degeneration. Upon cellular activation, microglia upregulates the synthesis and secretion of a number of proteolytic enzymes including but not limited to MMP and elastase that are potentially involved in degradation of the ECM and direct lytic attack of neurons (Wood, 2002). TNF α , IL-18, IL-6, or IL-23 act on neural cells and on peripherally derived immune cells during neuroinflammation. In addition, they release ROS and nitrogen species, which are

toxic to oligodendrocytes and neurons. Microglia also induces neurotoxic astrocytes by secreting IL-1 α , TNF α , and the complement component C1q. Disease-associated microglial phenotypes with specific transcriptomic signatures can be detected in AD and MS brains (Prinz et al., 2019).

The mechanisms that regulate the microglial phenotype in disease have not been entirely identified. The phenotypic transformation from homeostatic microglia towards reactive microglia, initiated by specific ligand binding to pattern recognition receptors including toll-like receptor 4 (TLR4) (Kumar, 2019). Indeed, pro-inflammatory signaling pathways trigger the caspase-mediated immune response. LPS binding to TLR4 triggers sequential activation of caspase-8 and caspase-3 with nuclear translocation of NF- κ B and expression of genes involved in inflammatory response (Rodríguez-Gómez et al., 2020). In addition, it was shown that TREM2-APOE pathway (triggering receptors expressed on myeloid cells-2 (TREM2), induces a microglia phenotypic switch from a homeostatic to neurodegenerative phenotype. A recent study has identified a role for apolipoprotein E (APOE) in regulating a subset of microglia, which exhibits a common disease-associated phenotype. This neurodegenerative phenotypic switch is triggered by TREM2, leading to activation of APOE signaling and subsequent suppression of homeostatic microglial phenotype. A functional consequence of the activation of TREM2-APOE pathway is switch of microglia to neurodegenerative phenotype thus lose the ability to regulate brain homeostasis (Krasemann et al., 2017).

Furthermore, new research disciplines such as epigenetics have provided evidences that the epigenetic mechanisms are also responsible for mediating environmental alterations in immune homeostasis by affecting microglia. It is noteworthy that major epigenetic mechanisms include post-translational modifications of histones, DNA methylation at CpG dinucleotides, and regulation by non-coding RNAs (Carrillo-Jimenez et al., 2019).

In addition, recently it has been demonstrated that dysregulation of energy metabolism in microglia, even minor alterations, relate to the progression of neurodegenerative disorders such as AD and PD (Edison et al., 2013). Indeed, modulating the cellular metabolism influences phenotypic changes of microglia in both directions: activation and deactivation. Specifically, inhibition of the glycolysis dramatically reduces responses of microglia to A β -induced

inflammation, whereas boosting glycolytic metabolism with IFN- γ restores microglial processes motility and increases A β phagocytosis (Baik et al., 2019). Metabolic alterations in microglia metabolic pathways can be associated with neurotoxic effects, production of IL-1 β , wound healing and NO production (Rodríguez-Gómez et al., 2020) as well as enhancement of Ten-eleven translocation 2 (TET2) upon stimulation by the NF- κ B-dependent pathway (Carrillo-Jimenez et al., 2019). The TET2 regulates early gene transcriptional changes, leading to early metabolic alterations in microglia. Microglia can exhibit widely differing functions at different stages in life both physiological and in response to, and in contribution to, various pathological situations are discussed greatly in reference (Boche et al., 2013).

Microglia, just as macrophages, are phagocytes of the CNS, capable of engulfing microorganisms, whole neurons or even parts of blood vessels depending on their different activation states, as governed by the antigen they encounter (Dudvarski Stankovic et al., 2016). In several CNS diseases the dualistic roles of distinctly polarized macrophage populations have been reported. Recently, the concept of microglial M1- and M2- phenotypes also has entered the field of stroke research (**Fig.16**). In addition, microglial cells are gate-keepers of the brain regulators of the growing vasculature, classic pro-inflammatory M1 type being characterized by an enhancement of proinflammatory cytokines (e.g., TNF α , IL-1 β , and IL-12), chemokines (IP-10, CCL5, and CXCL10), as well as inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), which are under tight regulation by NF- κ B in resting microglia (Ganbold et al., 2020). M1 is implicated in pro-inflammatory defense, tissue damage and tumor resistance (Dudvarski Stankovic et al., 2016). Alternatively, microglia may assume an M2 phenotype, which is pro-angiogenic and immunosuppressive, induces effector T cell and demonstrates impairments in cytotoxicity. Such cells express anti-inflammatory cytokines, such as IL-10 and TGF- β . M2 is implicated in immunoregulatory, anti-inflammatory, allergic and anti-parasitic response, tumor promotion and extracellular matrix remodeling (Dudvarski Stankovic et al., 2016).

An *in vitro* study has shown that LPS activates microglia to produce ROS through NADPH oxidase, resulting in a dysfunction of the BBB characterized by a decreased transendothelial electrical resistance, an increase of permeability to sodium-fluorescein as well as a TJs complex

fragmentation in rat brain microvascular ECs co-cultured with microglia (Sumi et al., 2010). In microglia, activated NF- κ B components translocate into the nucleus and initiate the transcription of proinflammatory cytokines and the other potential neurotoxins implicated in inducing the increase in the BBB permeability and the extravasation of leukocytes (Dudvarski Stankovic et al., 2016; Ganbold et al., 2020). Based on the current knowledge, there appears to be four main ways by which BBB disruption and endothelial activity can drive microglia activation: 1) factors expressed by endothelial cells such as cytokines and chemokines, 2) systemic inflammagens extravasating across the leaky BBB, 3) factors derived from pericytes and ECM remodeling, and 4) the effect of a long-term BBB dysfunction on microglia (Thurgur and Pinteaux, 2019).

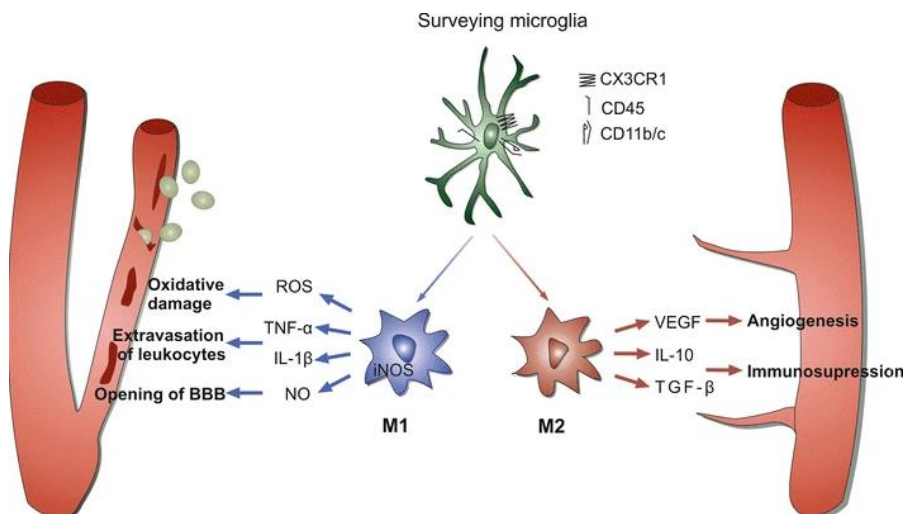


Figure 16. Impact of microglia polarization on blood vessels

Continuously surveying microglia palpate their microenvironment. Depending on the activation signal they encounter, they can differentiate into manifold activation states represented by two extreme phenotypes, namely the pro-inflammatory M1 state and anti-inflammatory M2 state. Among other functions, M1-like microglia promote the opening of the BBB followed by the extravasation of leukocytes, while M2-like cells secrete pro-angiogenic and immunosuppressive mediators. Adapted from (Dudvarski Stankovic et al., 2016).

1.9.1.3 Glial cell cross-talk in CNS inflammation

Reactive glial cells interact with each other and other cells in the CNS including neurons, endothelial cells as well as peripheral immune cells (Salvesen et al., 2018) and those bidirectional signaling events regulate CNS inflammation and pathological outcomes (Linnerbauer et al., 2020).

Microglia and astrocytes interact via contact-dependent and/or secreted factors (multiple cytokines and inflammatory mediators) to modulate their function during normal health and in disease. This bidirectional communication could induce positive or negative outcome (Jha et al., 2019).

A new role for activated microglia in inducing types A1-reactive astrocytes through the secretion of IL-1 α , TNF α , and C1q has been shown *in vitro* and *in vivo* (Liddel et al., 2017). It has been suggested that VEGF-B produced by microglia worsens the pathogenic activities of astrocytes in the experimental autoimmune encephalomyelitis (EAE) mouse model of multiple sclerosis (Rothhammer et al., 2018). Other investigation revealed that IL-1 β produced by astrocytes decreased about to 80% the production of NO by the inhibition of IFN- γ -induced production of NO in microglia (Tichauer et al., 2007). As already mentioned, NO at a basal concentration is capable of regulating numerous physiological functions (neuronal communication, vascular tone regulation etc.), and at higher concentrations can cause disease (cell death) (Ridnour et al., 2007). Upon activation, astrocytes release neurotoxic amounts of NO or glutamate, or they downregulate the uptake of extracellular neurotransmitters, ultimately leading to neuronal and oligodendrocyte death. Astrocytes furthermore control the recruitment of oligodendrocytes through the secretion of multiple cytokines. In addition, astrocytes respond to pro-inflammatory mediators secreted by activated oligodendrocytes (Linnerbauer et al., 2020). For example, among the multiple soluble factors released by microglia after LPS stimulation, the cytokine TNF α is one of the earliest released and most abundant pro-inflammatory factors which plays a pivotal role as a paracrine signal to regulate the neuroprotective functions of astroglial cells by production of astroglial GDNF (Chen et al., 2015).

Moreover, recent investigation demonstrated that LPS induced ROS and NO production in astroglia culture containing microglia but not in the microglia-depleted astroglia culture (Iizumi et al., 2016). Additionally, astrocytes can inhibit microglial activation by production of an acute-phase protein, orosomucoid (Jo et al., 2017). Astrocyte-derived lipocalin-2 mediates hippocampal damage and cognitive deficits in experimental models of vascular dementia, following increased microglial activity (Kim et al., 2017). One form of reactive astrocytes is directly mediated by C1q

complement, as well as IL-1 α and TNF α cytokine released by microglia; it appears to drive neuronal death and synapse density loss in mice (Dejanovic et al., 2018; Liddel et al., 2017). In addition, Bohlen *et al.*, have found that cholesterol, colony stimulating factor 1 (CSF1) /IL-34 and TGF- β 2 represent the three primary astrocyte-derived components that prevent *ex vivo* microglial death (Bohlen et al., 2017). Indeed, microglia requires cholesterol to maintain a physiological state, a lack of astrocyte-derived cholesterol is likely responsible for driving this microglial phenotype (Liddel et al., 2020).

1.9.2 Oxidative stress

Oxidative stress reflects an imbalance between oxidants and antioxidants in favor of the oxidants, leading to a disruption of redox signaling resulting in cell damage (Sies, 2015). Oxidative stress induced by generation of excess ROS which are highly reactive chemical molecules such as oxygen free radicals, e.g. superoxide anion radical ($O_2^{\bullet(-)}$), hydroxyl radical ($\bullet OH$), hydroperoxyl radical ($H-O-O \bullet$), singlet oxygen (represented as $^1\Delta gO_2$, abbreviated as 1O_2 , an excited state of O_2), as well as free nitrogen radicals such as nitric oxide (NO), peroxynitrite ($ONOO\bullet$), nitrogen dioxide (NO_2), called reactive nitrogen species (RNS) (Jakubczyk et al., 2020; Ozcan and Ogun, 2015). Among the different cells of an organism, brain cells are particularly vulnerable to oxidative damage due to their high energy-consumption and high metabolic rate (Busija and Katakam, 2014). High energy-consumption, high metabolic rate of the nervous tissue, high content of unsaturated lipids, metals of mixed valence (especially iron) and neuro-regulation by the free radicals (endogenous NO), increase the vulnerability of the brain to oxidative stress (Ermakov et al., 2021). Furthermore, 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) (Doll et al., 2015). Oxidative stress also induces inflammation (Phaniendra et al., 2015), which can further increase oxidative stress activity (Taylor et al., 2013).

ROS are derived from both endogenous (mitochondria, peroxisomes, endoplasmic reticulum, phagocytic cells etc.) and exogenous sources (pollution, alcohol, tobacco smoke, heavy metals, industrial solvents, pesticides etc) (Phaniendra et al., 2015). 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 ROS (Di Meo et al., 2016). It is noteworthy that the intrinsic apoptotic pathway is mediated by mitochondria through ROS production. Toxic effects of oxidative stress lead to aggregation or denaturation of cell proteins, and to peroxidation of lipids. Further, oxidative stress induces DNA damage in both the mitochondrial and nuclear genomes (Chen and Zhong, 2014).

The adult human brain constitutes approximately 2% of the total body weight, however, it consumes about 20% of the body's energy. In brain about 90% of the energy required for CNS function is provided by aerobic mitochondrial respiration (Tefera et al., 2021). Mitochondria, the best known for producing ATP via oxidative phosphorylation (OXPHOS) is comprised of two separate and functionally distinct outer and inner membranes that encapsulate the intermembrane space and matrix compartments. Cellular respiration is a multi-step process that all living things use to breakdown of glucose and other respiratory substrates into energy-providing molecules called adenosine triphosphate (ATP). Metabolic pathway involved in respiration can be divided into three main pathways (**Fig.17**). Glycolysis which occurs in the cytoplasm, the tricarboxylic acid (TCA) cycle which occurs in the matrix of the mitochondria and finally the electron transport chain (ETC) occurring in the inner membrane of the mitochondria (Adams and Turnbull, 1996; Angelova and Abramov, 2018). Briefly, in brain, glucose moves across the endothelial membrane into the extracellular fluid via facilitated transport by glucose transporter. Glycolysis metabolic pathway converts one molecule of glucose into two molecules of pyruvate. Pyruvate then is oxidized to acetyl-CoA and CO₂ by the pyruvate dehydrogenase complex that is located in the mitochondria of eukaryotic cells. Once acetyl-CoA is formed, mitochondria will undergo aerobic respiration which leads to the TCA cycle. TCA creates the electron carrier molecules NADH and FADH₂, along with ATP. The electron transport chain is the final step in cellular respiration. It uses the electron carrier molecules NADH and FADH₂ created during the TCA. The electron transport chain consists of four protein complexes (I–IV), which through sequential redox reactions undergo conformational changes to pump protons from the matrix into the intermembrane space. The proton gradient generated by complexes I, III, and IV is released through the rotary turbine-like ATP synthase machine or complex V, which drives phosphorylation of ADP to ATP (Iizumi et al., 2016; Tefera et al., 2021). In addition to their

powerhouse role, mitochondria also regulates cellular levels of metabolites, amino acids, and cofactors for various regulatory enzymes, and is involved in the synthesis of steroid hormones (Manoli et al., 2007). Moreover, mitochondria plays a central role in metal, mainly copper and iron, metabolism of heme and Fe-S clusters which are essential components of the major oxygen carrier, hemoglobin, as well as DNA repair machinery (Nunnari and Suomalainen, 2012).

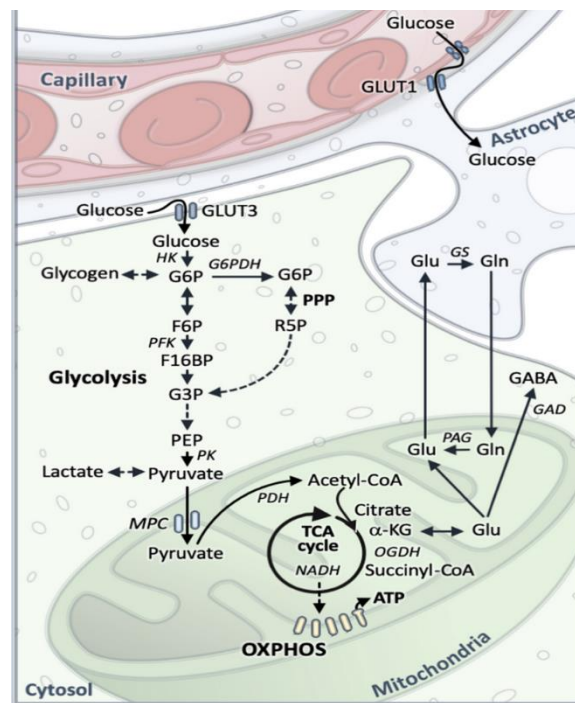


Figure 17. A simplified summary of CNS glucose metabolism

Glucose enters into neurons via glucose transporter (GLUT3) and via GLUT1 into astrocytes. Glycolysis takes place in the cytosol of cells, can happen with or without oxygen, and involves a series of 10 chemical reactions converting glucose to pyruvate. Pyruvate then is oxidized to acetyl-CoA. Once acetyl-CoA is formed, mitochondria will undergo aerobic respiration which leads to the TCA cycle. Acetyl-CoA condenses with oxaloacetate to citrate and thereby enters the tricarboxylic acid (TCA) cycle. In neurons, the TCA cycle generates different TCA cycle intermediates such as, α -ketoglutarate, a precursor of glutamate. Adapted from (Tefera et al., 2021).

Mitochondria also participate in Ca^{2+} homeostasis (Wang and Wei, 2017) shaping the spatiotemporal distribution of this second messenger by buffering Ca^{2+} flux from the plasma membrane and endoplasmic reticulum (Nunnari and Suomalainen, 2012).

Abnormalities in any step of these metabolism pathways can result in reduced generation of ATP, which may affect basic cellular functions. Also, since glucose metabolism is linked with amino acid neurotransmitter metabolism, abnormalities in glucose metabolism pathways may

contribute to glutamate excitotoxicity and neurodegeneration (Tefera et al., 2021). Indeed, glutamate which is synthesized from α -ketoglutarate (α -KG, a TCA cycle intermediate), is released during neurotransmission from the presynaptic vesicles into the synapse where it is taken up by astrocytic glutamate transporters and then is converted into glutamine by glutamine synthetase (GS). Glutamine can also be transferred into neurons and gets converted into glutamate by phosphate activated glutaminase (PAG), completing the glutamate-glutamine shuttle. In GABAergic neurons, glutamate is converted into GABA by glutamate decarboxylase (GAD) enzyme (Fig.17).

In several CNS dysfunction, numerous metabolic defects at various steps in the glucose metabolism pathway that alter glucose metabolism and overall ATP generation, have been described (Song et al., 2020). In the brain, the main glucose transporters (GLUTs) are GLUT3 in neurons and GLUT1 in the microvascular ECs of the BBB and glia. The intracellular signaling pathways involved in GLUT regulation in cerebral ischemia remain unclear; however, it has been established that ischemia induces changes in their expression (Monica et al., 2010). In addition, oxidative damage to enzymes involved in glycolysis, the tricarboxylic acid cycle and ATP biosynthesis in AD has been established. The glycolysis impairment affects ATP-requiring processes for cognitive and synaptic function and neuronal integrity (Butterfield and Halliwell, 2019). Further recent investigation revealed that oxidative stress increased glycogen accumulation in astrocytes upon cerebral reperfusion and activating glycogenolysis protects against acute and subacute reperfusion insult (Cai et al., 2020). Beside, reduced pyruvate entry into the TCA cycle in epilepsy was documented (McDonald et al., 2018). Altered neuron-glia interaction represents another outcome of stress oxidative in most the often neurodegeneration diseases (Mulica et al., 2021).

It was shown that a decline in ATP levels can lead to a compensatory activation of other energy generating pathways, which may increase production of ROS and worsen the oxidative stress state (Tefera et al., 2021). Dysfunction in cellular respiration, particularly in the electron transport chain could be a reason for mitochondrial dysfunction. The main primary mechanisms leading to the triggering of oxidative stress involve the formation of ROS and mitochondrial

dysfunction. Some secondary mechanisms include excitotoxicity, iron metabolism and inflammation (Song et al., 2020).

ROS production in low amount during physiological situations have beneficial effects in many cellular signaling pathways including cell proliferation, activation, migration, etc., while high amount production of oxidative species occurring under pathological situations have a particularly adverse effect in cancers (Kudryavtseva et al., 2016) neurological (Song et al., 2020) and inflammatory diseases (Kattoor et al., 2017).

The main source of ROS production within most cells is the mitochondria. The mitochondria are not only the major site of intracellular ROS production but are also the main target organelle of ROS-induced injury. Within the mitochondria, the primary ROS produced is superoxide which is the precursor of most other ROS due to its conversion to hydrogen peroxide by the action of superoxide dismutase (Dan Dunn et al., 2015). Mitochondrial proton and electron leak have a major impact on mitochondrial coupling efficiency and production of ROS (Jastroch et al., 2010). The sites of electron leak causing superoxide production in the respiratory chain, are considered to be a semiquinone radical or a reduced flavin. Thus, Complex I as well as both sides of complex III are the most common sources of mitochondrial superoxide. At complex I, superoxide may be either generated at the flavin or at the IQ site and is completely released to the matrix side. At complex III, superoxide is produced at the Qo site and released to intermembrane space and matrix (Jastroch et al., 2010). During ischemia, the superoxide generated within the intermembrane space of mitochondria can reach the cytosol, then be increased by cyclooxygenase-2 (COX-2), NADPH oxidase, uncoupled eNOS, and infiltrating leukocytes (**Fig18**). The resulting high levels of superoxide, coupled with the activation of NO-producing eNOS/iNOS, increases the likelihood of peroxynitrite formation. Peroxynitrite-induced cellular damages include protein oxidation, tyrosine nitration, DNA damage, and poly(ADP-ribose) polymerase (PARP) activation, lipid peroxidation, and mitochondrial dysfunction (Abdullahi et al., 2018). ROS and reactive nitrogen species (RNS) play pivotal roles in neuronal damage. ROS and/or RNS are, at least in part, produced by cerebral parenchymal cells, including neurons and glial cells, as well as peripheral inflammatory cells such as leukocytes. The origin of ROS appears to be from

activated NADPH oxidase. ROS, in turn, reacts with NO produced by activated iNOS, leading to peroxynitrite generation, which is a highly toxic reactive species that can induce lipid peroxidation and protein oxidative damage (Iizumi et al., 2016). NO can deleteriously alter the activities of enzymes such as Cyt-C oxidase and NAD dehydrogenase (**Fig18**).

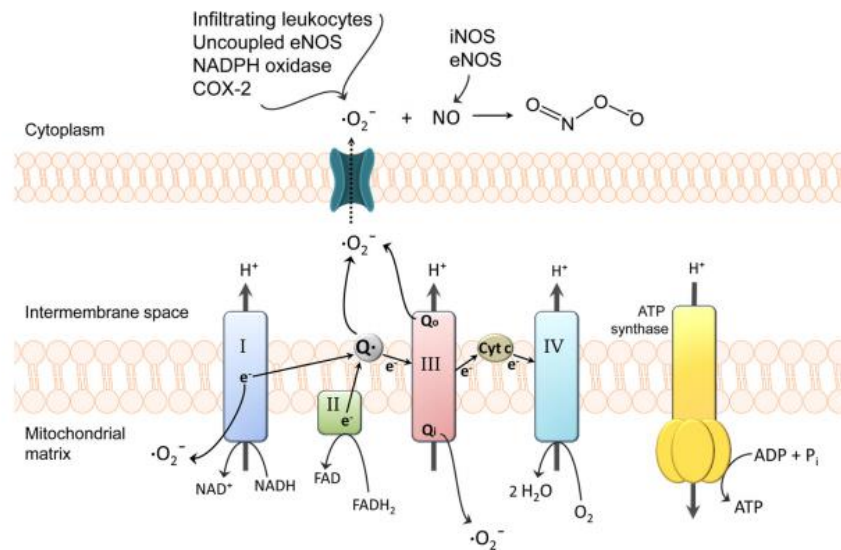


Figure 18. Generation of reactive oxygen species in cerebrovascular endothelial cells during ischemia

Adapted from, (Abdullahi et al., 2018).

Increasing evidence shows that mitochondria are key players in BBB permeability and oxidative stress, with an essential role in the induction of BBB changes (Doll et al., 2015). ROS-related pathways that trigger BBB dysfunction include excitotoxicity, mitochondrial dysfunction, microglial activation, extracellular transport, TJ modification, and MMP activation (Song et al., 2020). It has been shown that the cerebral capillaries exhibiting the BBB characteristic, show high mitochondrial content (8 to 11% of the endothelial cytoplasmic volume) while in the regions known to be devoid of BBB (for example, anterior pituitary and median eminence), the mitochondrial volume is 2 - 5% of their respective cytoplasmic volume (Oldendorf et al., 1977). This greater number of mitochondria may be related to the excess metabolic work capability of the BBB to maintaining brain hemostasis. It was shown that instances of hypometabolism of glucose and oxygen in the brain induced by pathologies and non-homeostasis cause mitochondrial dysfunction, energetic failure and oxidative stress (Mulica et al., 2021).

Mitochondria abnormalities in endothelial cells may contribute to cerebrovascular dysfunction, commonly present in stroke (Sims and Muyderman, 2010), Alzheimer and other neurodegenerative disorders (Islam, 2017)

ROS contribute to BBB disruption by several mechanisms: oxidative damage of cellular molecules (proteins, lipids and DNA), activation of matrix metalloproteinases (MMPs), cytoskeletal reorganization, modulation of TJ proteins and upregulation of inflammatory mediators (Pun et al., 2009). Oxidative stress participates in the development of AD by promoting A β deposition, tau protein hyperphosphorylation, and the subsequent loss of synapses and neurons (Chen and Zhong, 2014).

1.9.3 Matrix metalloproteinases

MMPs are calcium-dependent zinc-containing endopeptidases, belonging to proteases family. Currently there are 24 known MMPs identified in vertebrates, including 23 in humans. (Rempe et al., 2016).

Based on the organization of their structural domains and substrate specificity, they were divided into six groups collagenases (MMP-1, -8, and -13), gelatinases (MMP-2 and -9), stromelysins (MMP-3, -10, and -11), matrilysins (MMP-7 and -26), membrane-type MMPs (MMP-14, -15, -16, -17, -24, and -25) and other non-classified MMPs (MMP-12, -18, -19, -20, -21, -22, -23, -27, and -28) (Massova et al., 1998). A typical MMP consists of a propeptide, a catalytic metalloproteinase domain, a linker peptide (hinge region), and a hemopexin domain (**Fig.19**). The propeptide has a cysteine switch PRCGXPD which cysteine sulfhydryl (–SH) group chelates the active site Zn²⁺, keeping the MMP in the latent zymogen proMMP form. The catalytic domain contains the Zn²⁺ binding motif HEXXHXXGXXH, two Zn²⁺ ions (one catalytic and one structural), specific S1, S2, ...Sn and S1', S2', ...Sn' pockets, which confer specificity, and two or three Ca²⁺ ions for stabilization. Some MMPs show exceptions in their structures. Gelatinases have 3 type-II fibronectin repeats in the catalytic domain. Matrilysins have neither a hinge region nor a hemopexin domain. Furin-containing MMPs such as MMP-11, 21 and 28 have a furin-like pro-protein convertase recognition sequence in the propeptide C-terminus. MMP-28 has a slightly

different cysteine switch motif PRCGVTD. Membrane-type MMPs (MT-MMPs) typically have a transmembrane domain and a cytosolic domain. MMP-17 and -25 have a glycosylphosphatidylinositol (GPI) anchor. MMP-23 lacks the consensus PRCGXPD motif, has a cysteine residue located in a different sequence ALCLLPA, may remain in the latent inactive proform through its type-II signal anchor, and has a cysteine-rich region and an immunoglobulin-like proline-rich region (Cui et al., 2017).

MMPs are expressed as inactive zymogens with a pro-peptide domain (pro-MMPs) that must be removed for MMP activation. The proteolytic properties of MMPs are controlled by the tissue inhibitors of metalloproteinases (TIMPs) and the MMP/TIMP ratio often determines the extent of ECM protein degradation and tissue remodeling. The expression of TIMP is regulated by the cytokines and growth factors (Jabłońska-Trypuć et al., 2016). MMPs may serve as biomarkers and potential therapeutic targets for certain vascular disorders.

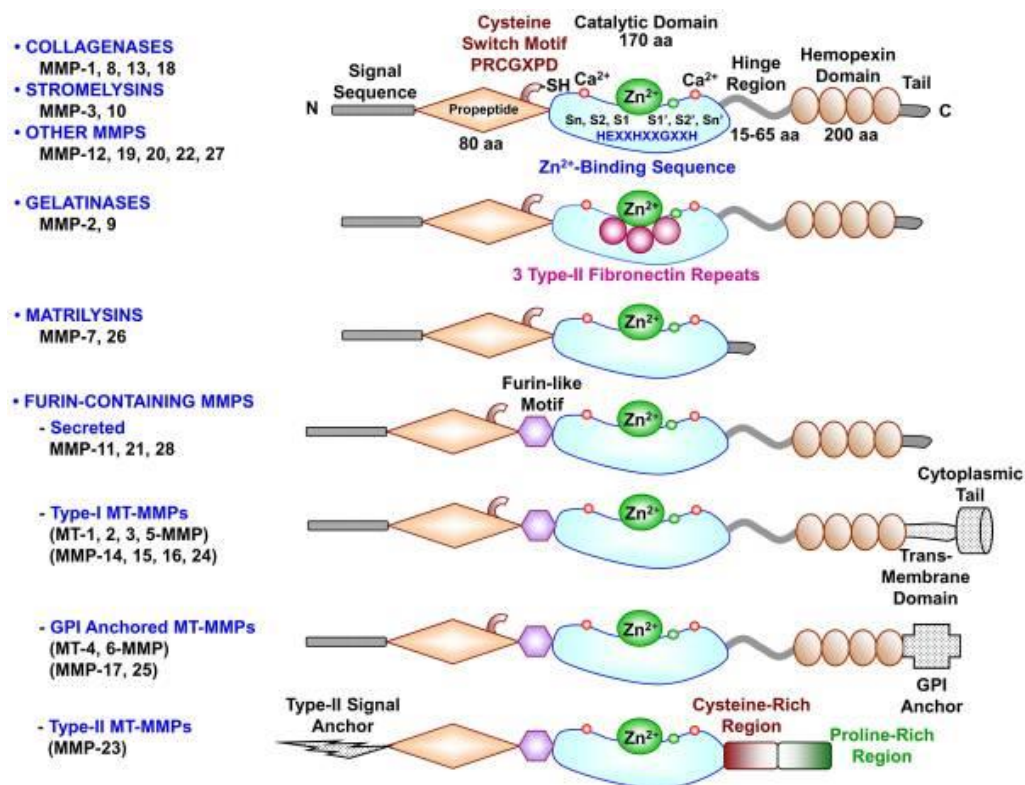


Figure 19. Major MMPs subtypes and their structure

Adapted from, (Cui et al., 2017).

MMPs are produced by multiple tissues and cells including connective tissue, pro-inflammatory and uteroplacental cells including fibroblasts, osteoblasts, endothelial cells, vascular smooth muscle, macrophages, neutrophils, lymphocytes, and cytotrophoblasts (Wang and Khalil, 2018). MMPs participate in several physiological processes in both developing and adult organism, including but not limited to, CNS development and morphogenesis, cell proliferation and migration, differentiation of cell precursors and angiogenesis (Agrawal et al., 2008). MMPs play a role in vascular tissue remodeling during various biological processes such as angiogenesis, embryogenesis, morphogenesis and wound repair (Wang and Khalil, 2018). These biological processes take place multiple times during normal development and organogenesis, but, if not properly balanced, could also contribute to harmful pathological processes (**Fig.20**) including wound healing, inflammation, vascular diseases (such as atherosclerosis, aneurysms, and chronic venous diseases) and, tumor metastasis and cancer (Agrawal et al., 2008; Rempe et al., 2016).

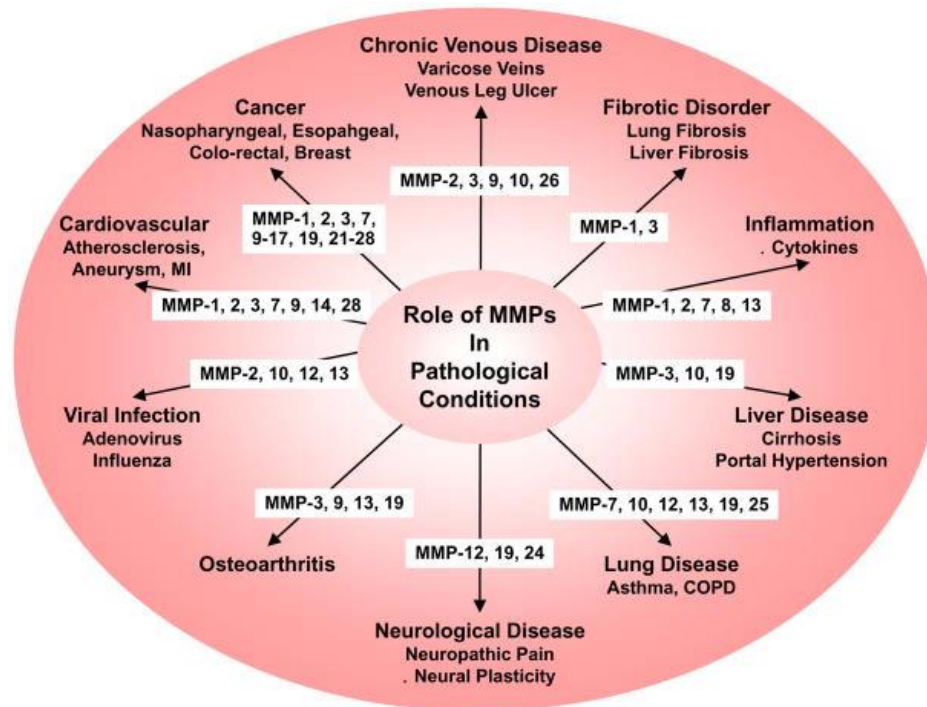


Figure 20. Representative roles of MMPs in pathological conditions.

COPD, chronic obstructive pulmonary disease; MI, myocardial infarction. Adapted from, (Cui et al., 2017).

Alterations in specific MMPs could influence arterial remodeling leading to various pathological disorders such as hypertension, preeclampsia, atherosclerosis, aneurysm formation, as well as excessive venous dilation and lower extremity venous diseases (Wang and Khalil, 2018). MMPs can participate in these processes by several mechanisms including the proteolytic cleavage of growth factors so that they become available to cells that are not in direct physical contact, degradation of ECM components so that founder cells can move across the tissues into nearby stroma, and the regulation of receptor cleavage in order to terminate migratory signaling and cell migration (Cui et al., 2017).

MMPs and their inhibitors, TIMPs, have a role in the dynamic regulation of the BBB not only in physiological but also in inflammatory conditions. Undesirable actions of abnormally upregulated MMPs include disruption of the BBB, and promotion of neuroinflammation. MMPs are known as a group of enzymes that regulate cell–matrix composition (McCawley and Matrisian, 2001) and in a pathological condition it has been shown that MMPs cleave ECM proteins to allow infiltrating leukocytes, metastatic, and transformed cells, to penetrate ECM barriers (Lukes et al., 1999; Rempe et al., 2018). Further impairment of MMP-2 and TIMP-2 levels induces the degradation of collagen type IV leading to BBB dysfunction in pathogenesis of brain injury (Lee et al., 2012). In the pathogenesis of schizophrenia, MMP-mediated ECM abnormality thereby plays a detrimental role in synaptic functions and BBB patency (Chopra et al., 2015). It was shown that IL-1 β -induced MMP-9 secretion in pericytes contributes to BBB impairment in neuroinflammatory diseases by disruption of VE-cadherin, occludin, claudin-5, and ZO-1 (Qin et al., 2019). MMP-2 and MMP-9 have been shown to mediate claudin-5 and occludin degradation, and play an important regulatory role in BBB permeability. In the traumatic brain injury, there is an altered expression of MMP9 associated with degradation of TJs coupled with an increased BBB permeability (Main et al., 2018). In addition, the autocrine secretion of MMP-2/9 by cerebral ECs increases BBB permeability by upregulation of VCAM, thereby facilitating the entry of inflammatory cells into the central nervous system (Tasaki et al., 2014). MMPs have been implicated in pathophysiology of cerebral ischemia and treatment with an MMP inhibitor resulted in significantly lower lesion volumes compared to injection of vehicle (Klohs et al., 2009). In a rat stroke model of transient middle cerebral artery occlusion (MCAO), increasing NO concentrations during reperfusion

activate MMP-2 and MMP-9 by downregulation of caveolin-1 which is also a negative regulator of NO-synthases (NOS) (Gu et al., 2012); blocking NOS can reversed these downstream effects (Obermeier et al., 2013). Beside in the context of neuro-inflammatory diseases, MMPs have been involved not only in the BBB opening but also in invasion of neural tissue by blood-derived immune cells, and alteration of cytokines and cytokine receptors (Leppert et al., 2001).

1.9.4 Physiological aging

Physiological aging involves a progressive detrimental change in maximal organ function with differential trajectories across organ systems (Khan et al., 2017). Decline of in neural function that contributes to cognitive impairment and age related pathologies, represents the main health challenge of aging (Costea et al., 2019). Although, mechanisms underlying the transition from a young-and-healthy to aged-and-dysfunctional brain are not well understood however neural function decline is mechanistically linked to the state of the microvasculature in the brain (Senatorov et al., 2019). Alterations of BBB during physiological aging concern all constituents of the NVU such as alteration of thickness and number of ECs, decrease of the expression of tight junction proteins, alteration of thickness and composition of the basement membrane, activation of glial cells or production of neurotoxic proinflammatory mediators, the decrease in the number of pericytes or pericytes detachment, and neuronal damage (Erdő et al., 2017).

A recent study has interrogated the RNA profile of the BBB in an ageing human brain cohort after autopsy and an ageing mouse model, using combined laser capture microdissection and expression profiling (Goodall et al., 2019). They observe that 12 overlapping genes are altered in the same direction in the BBB of both ageing human and mouse cohorts. These include genes with roles in regulating vascular tone, tight junction protein expression and cell adhesion proteins, all processes prone to a dysregulation with advancing age. They also identify 15 overlapping miRNAs that have showed altered expression. In addition to targeting genes related to DNA binding and/or autophagy, many of the miRNAs identified play a role in age-relevant processes, including BBB dysfunction and regulation of the neuroinflammatory response. (Goodall et al., 2019).

The continued advancement of transcriptomic profiling techniques, including microarray analysis, has enabled the identification of specific gene expression changes and biological processes associated with ageing. A transcriptional profiling of the human frontal cortex from individuals ranging from 26 to 106 years of age, defines a set of genes with reduced expression after age 40. (Lu et al., 2004). The cluster of age-downregulated genes includes many genes that play integral roles in synaptic plasticity, including NMDA and AMPA receptor function, calcium-mediated signaling, and synaptic vesicle release and recycling. In addition, the reduced expression of key calcium-binding and homeostatic genes in the aged cortex could compromise intraneuronal calcium homeostasis, and may increase neuronal vulnerability to toxic insults (Lu et al., 2004). It is important to note that gene expression profile assessment reveals two overarching concepts. First, different regions of the forebrain exhibit substantially different gene profile changes with age and second, there is an obvious gender differences in brain aging, suggesting that the brain undergoes sexual dimorphic changes in gene expression not only in development but also in aging. Globally, across all brain regions, males show more gene change than females. Notably, the male brain is characterized by global decreased catabolic and anabolic capacity with aging, with down-regulated genes heavily enriched in energy production and protein synthesis/transport categories (Berchtold et al., 2008). However, an increase of immune activation is a prominent feature of aging in both sexes, with proportionally greater activation in the female brain. The mechanisms responsible for the sexually dimorphic patterns in gene expression profiles in brain aging are undoubtedly multifaceted. One of the strongest environmental risk factors for a host of neurodegenerative conditions, is age. BBB dysfunction and vascular impairments have also been identified in the pathophysiology of many of these diseases. Such gender-specific tissue and cellular differences likely become amplified with age (Berchtold et al., 2008).

A recent evidence has suggested that there is a decreased vascular density in ageing, AD and leukoaraiosis, and cerebrovascular dysfunction precedes and accompanies cognitive dysfunction and neurodegeneration. A decline in cerebrovascular angiogenesis may inhibit recovery from hypoxia-induced capillary loss. Cerebral blood flow is inhibited by tortuous arterioles and deposition of excessive collagen in veins and venules (Brown and Thore, 2011).

A meta-analysis of 31 BBB permeability study (1953 individuals) in normal ageing or in cerebral microvascular disease found in healthy humans, shows that BBB permeability increases with normal ageing (Farrall and Wardlaw, 2009). In addition, other data suggest that BBB breakdown is an early event in the aging human brain that begins in the hippocampus and may contribute to cognitive impairment (Montagne et al., 2015). CSF analyses also showed that levels of $\text{SPDGFR}\beta$, a potential biomarker of pericytes injury, increases with age (Keaney and Campbell, 2015).

1.10 Cross-talk between age, gender and sex steroid hormones

Sex hormones, also known as sex steroids, gonadocorticoids and gonadal steroids, are steroid hormones that interact with steroid hormone receptors. Sex hormones include three major classes of steroids: androgens, estrogens, and progestogens (Larson, 2018).

Androgens (from the greek “andro” meaning male or man) are traditionally considered as male sex steroids responsible for the development and maintenance of male reproductive tissues and their characteristic properties. (Rommerts, 1990). Testosterone is the primary androgens and is produced by the Leydig cells of the testes (Li et al., 2018a). Dihydrotestosterone (DHT), a potent metabolite of testosterone is of equal importance in male development. Testosterone is synthesized by both males and females, but in different quantities (Vodo et al., 2013); the production by the testes of males is 7 to 8 times higher than that produced by the ovaries in females (Hammes and Levin, 2019). The extragonadal biosynthesis of testosterone has been reported in many tissues, this includes the brain (local synthesis of neurosteroids has been reported in several cerebral regions such as the hippocampus) and liver in both males and females. In addition, the adrenal cortex zona reticularis and zona fasciculata in both sex produce the testosterone precursor steroids dehydroepiandrosterone (DHEA) and androstenedione (Gonzales, 2013). Also, androgens are the precursors to estrogens in both male and female. Estrogens were delineated as female-specific hormone as a result of their pronounced synthesis in ovaries. Estrogens control the development and maintenance of female sexual characteristics. The three major types of endogenous estrogens are estrone (E_1), 17β -estradiol (E_2), and estriol (E_3), with E_2 being the most prevalent and potent form of circulating estrogen (Larson, 2018). The

extra-gonadal biosynthesis of E₂ in adipocytes, brain (hippocampus and hypothalamus), spleen, blood vessels, intestines, liver and bone has been documented (Barakat et al., 2016). A third class of sex steroids, progestins, includes progesterone which is synthesized from pregnenolone in the corpus luteum of the ovaries and in the adrenal cortex. In addition progesterone metabolism in the pineal gland, brain stem, thalamus and corpus callosum has been reported (Hanukoglu et al., 1977).

Indeed, aging in men is accompanied by a progressive lowering of the serum testosterone level, in parallel with a decline in several CNS functions (Lecce et al., 2014; Lynch et al., 2010; Sandoval and Witt, 2011; Thibaut, 2016). This provides a logical reason to increase research to determine whether testosterone acts as a protective factor against several CNS disorders (Mahmoud et al., 2016). 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 and Parkinson's disease) diseases, with a higher incidence in females than in males (Mahmoud et al., 2016; Spence and Voskuhl, 2012). The reasons for gender differences, and a better understanding of how the state of being male limits these CNS disorders, are still unclear and even controversial. Gender differences are probably a composite of hormonal elements, environmental factors, and overall lifestyle components that affect overall health. Sex differences are not only determined by gender-related differences in sex steroid levels, but also by gender-specific tissue and cellular characteristics that mediate sex-specific responses to a variety of stimuli. Such gender-specific tissues and cellular differences likely become amplified with age (Berchtold et al., 2008). It appears that sex bias is partly due to the protective effect of testosterone, or to the presence of the Y chromosome in males (Ngun et al., 2011; Vodo et al., 2013). 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 pathology (Robison et al., 2019).

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 systems (Larson, 2018), including cerebrovascular tissue (Atallah et al., 2017a; Honda et al., 1999; Witt and Sandoval, 2014). Outside the brain, testosterone has a key effect on the cardiovascular system by the regulation of the vascular tone (Orshal and Khalil, 2004). 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 receptors (Bielecki et al., 2016). Nevertheless, the role of testosterone in vascular contractility is debated. A number of studies have shown in rat that testosterone facilitates vasoconstriction (*in vivo* and *in vitro*) (Ceballos et al., 1999; Masuda et al., 1991) while other data have reported a vasodilator effect in rat and rabbit (Costarella et al., 1996; Yue et al., 1995). Vascular mechanisms of testosterone have been thoroughly reviewed very recently (Lorigo et al., 2020).

2 Chapter 2: Sex steroid hormones signaling and male central nervous system

2.1 Biosynthesis of testosterone

Testosterone is a sex steroid hormone from the androgen group and the main male sex hormone. In mammals, testosterone is secreted mainly by the gonads, i.e., the testes in males and the ovaries in females, but to a lesser degree; the adrenal glands and some other tissues also produce testosterone in smaller amounts. Testosterone secretion is regulated by the hypothalamic-pituitary-gonadal (HPG) axis. In males, the pulsatile release of Gonadotropin-Releasing Hormone (GnRH) from the hypothalamic region induces the release of Follicle Stimulating Hormone (FSH) and luteinizing hormone (LH) from gonadotrope cells of the pituitary gland into the systemic circulation. The FSH acts to stimulate the activity of Sertoli cells in testicular seminiferous tubules to aid in spermatogenesis. LH, is the key hormone that activates testosterone synthesis in Leydig cells of testes through a G-protein-associated seven-transmembrane receptor with an uncommonly long extra-cellular domain (Winters and Clark, 2003) (**Fig.21A**).

Testosterone, like other steroid hormone, is produced from cholesterol transferred from intracellular stores into mitochondria (Li et al., 2018a). The access of cholesterol to the mitochondria is regulated by the steroidogenic acute regulatory protein, StAR, serving as the acute regulator of steroidogenesis (Miller, 2013) (**Fig.21B**).

LH binding triggers a signaling cascade by activating Gs (small GTPase) protein that stimulates adenylate cyclase activity to increase levels of intracellular cAMP and activate cAMP-dependent protein kinase A (PKA). cAMP-dependent PKA activates two temporally distinct responses that stimulate testosterone synthesis. The acute response, which occurs within minutes of hormonal stimulation, is defined as the stimulated increase in cholesterol transport into the mitochondria. The chronic response, which requires several hours, involves transcriptional activation of the genes encoding the steroidogenic enzymes of the testosterone

biosynthetic pathway (Winters and Clark, 2003). In mitochondria, cholesterol is metabolized by the cytochrome CYP11A1 into pregnenolone, which is further metabolized to androgens in the endoplasmic reticulum by the 3β -HSD, CYP17A1, and 17β -HSD3 enzymes. Testosterone can be metabolized to estradiol and dihydrotestosterone (DHT) by CYP19A1 and 5α -reductase, respectively (Li et al., 2018a) (**Fig.21B**). According to a new hypothesis DHT is converted to the estrogen receptor β binding molecule, 3β -Diol (5α - androstan- 3β , 17β -diol) by the combined action of the enzymes- 3α -hydroxysteroid dehydrogenase (3α -HSD), 3β -hydroxysteroid dehydrogenase (3β -HSD), or 17β -hydroxysteroid dehydrogenase (17β -HSD).

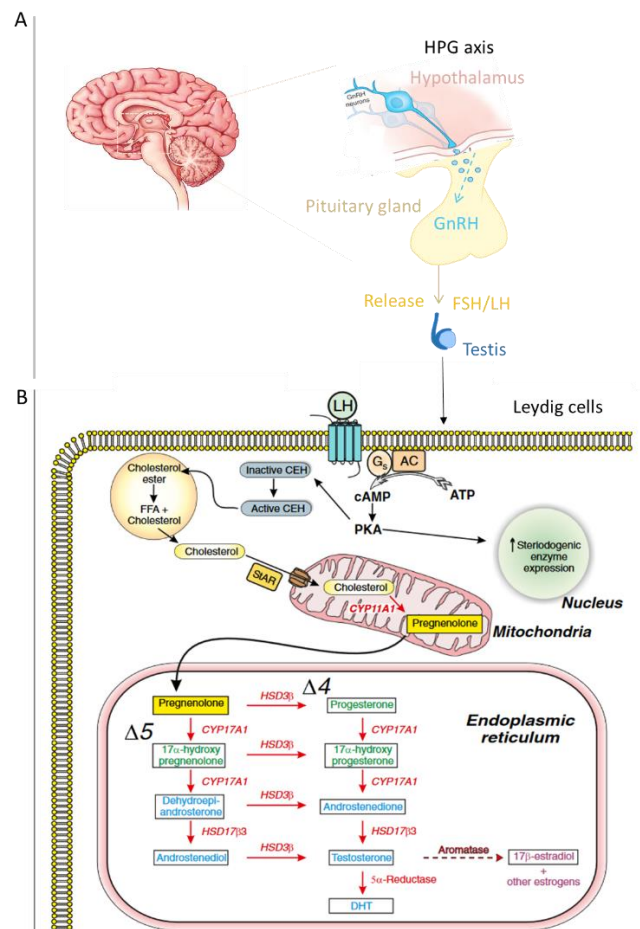


Figure 21. Testosterone biosynthesis and regulation

GnRH secreted from the hypothalamus releases LH from the pituitary. LH binds to LH receptors on Leydig cells, stimulates G_s , and activates the cAMP/ PKA pathway. PKA promotes the transport of cholesterol into mitochondria and increases transcription of genes involved in testosterone biosynthesis. Cholesterol is converted to pregnenolone, which diffuses into the endoplasmic reticulum for testosterone biosynthesis via $\Delta 4$ and $\Delta 5$ pathways. Testosterone is formed by 17β -hydroxysteroid dehydrogenase 3 (HSD17B3) in the $\Delta 4$ pathway and by 3β -

hydroxysteroid dehydrogenase (HSD3 β) in the $\Delta 5$ pathway. Testosterone is converted to dihydrotestosterone (DHT) by 5 α -reductase, and some are aromatized to 17 β -estradiol. Figure adapted and modified from (Ayaz and Howlett, 2015).

The actions of 3 β -Diol can be reduced by their further metabolism to the inactive compounds, 6-, or 7- triol by the enzyme cyp7b1. The molecule, 3 α -Diol undergoes a bidirectional conversion from DHT by 3 α -HSD and as such can act as a sink for further production of DHT (Handa et al., 2008) (**Fig.22**). If testosterone plasma levels rise too high, a negative feedback mechanism is then activated. Indeed, testosterone regulates its own production by negative feedback, suppressing LH secretion and downregulating gonadotropin subunit gene expression (Winters and Clark, 2003).

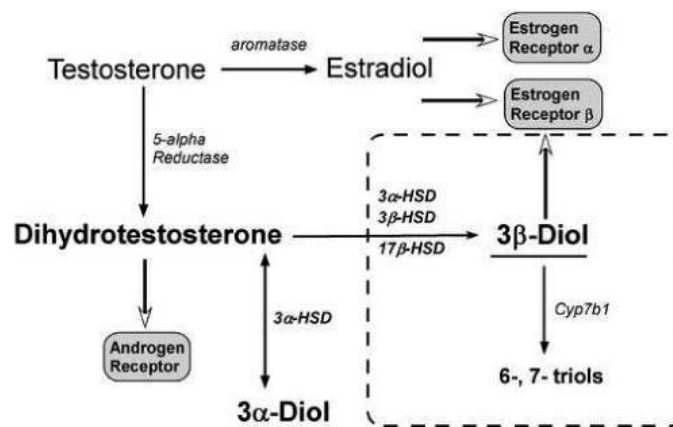


Figure 22. Diagram depicting central dogma concerning the actions of testosterone

Figure adapted from, (Handa et al., 2008).

2.2 Organizational and activational effect of testosterone

The actions of sex steroids on brain and behavior traditionally have been divided into organizational and activational effects. Organizational effects are permanent and occur early in development, while activational effects are transient and occur throughout life (Arnold and Breedlove, 1985). The organizing hormonal signal is the perinatal androgen surge from the testes, which begins prenatally. In rodent, after Leydig cell differentiation around embryonic day 12.5-13 (ED) testosterone production begins and quickly achieves a peak around ED 17-18 and then declining again, peaks again on the day of birth and rapidly declines within hours. At this stage, testosterone is responsible for masculinization and defeminization of urogenital tract and

masculinization and defeminization of the brain circuitry that controls sexually differentiated behaviors and reproductive physiological functions (Arnold and Breedlove, 1985; Konkle and McCarthy, 2011). After the neonatal testosterone peak, circulating testosterone levels decline to low levels, where they persist until the onset of puberty. Testosterone levels achieve their adult levels around PND 60, which is around 1-2 ng/ml (Arnold and Breedlove, 1985; Jean-Faucher et al., 1978; O'Shaughnessy et al., 2009). The activational effects occur during the adult period and at this stage, testosterone is responsible for appearance and maintains secondary sexual characteristics and spermatogenesis (**Fig.23**) (Konkle and McCarthy, 2011).

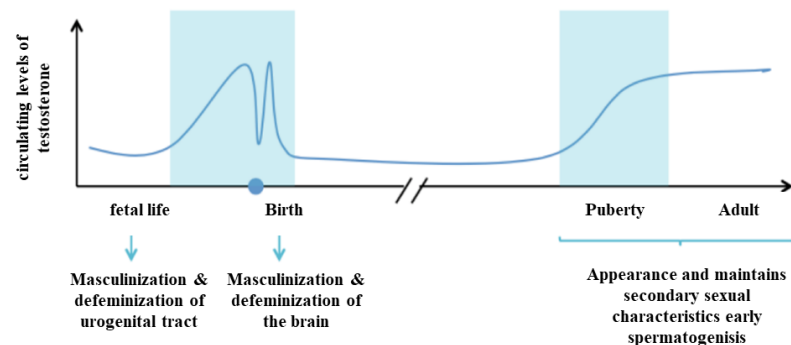


Figure 23. Variations in testosterone levels during life

Regions highlighted in blue correspond to critical periods of sensitivity. The perinatal period includes two peaks of testosterone, before and after birth, allowing masculinization and defeminization of the urogenital tract and neural circuits. The concentration of testosterone falls and remains low until puberty where it goes back allowing the acquisition of secondary sexual characteristics, the onset of spermatogenesis and the maturation of neuronal circuits (Konkle and McCarthy, 2011).

Sex steroids mediate their action via members of the nuclear receptor superfamily. Those are expressed widely throughout the CNS by multiple cell types, but especially in brain areas involved in cognitive and reproductive behavior, and in neuroendocrine function (Mhaouty-Kodja, 2018a).

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

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

E2 (Bennett et al., 2010; Larson, 2018). 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 and is only an androgen receptor ligand (Melcangi et al., 1998; Patchev et al., 2004).

To describe sex steroid receptor localization within the adult CNS, mRNA expression patterns of ARs and ERs were investigated in adult rats (**Fig.24**). (Patchev et al., 2004; Shughrue et al., 1997; Simerly et al., 1990). 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 behavior (Patchev et al., 2004). 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 endothelium (Gonzales et al., 2007).

Neural estrogens exert their effects through interaction with two nuclear ERs: estrogen receptor α (ER α) and β (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 (Patchev et al., 2004; Shughrue et al., 1997). 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 (Mhaouty-Kodja, 2018a; Patchev et al., 2004; Shughrue et al., 1997) (**Fig.24**). 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 HPG axis (Lauber et al., 1991). In addition to their neural expression, specific receptors for gonadal steroids are expressed in the cerebrovascular tissue (Gonzales et al., 2007). Additionally to their specific receptors, the metabolic enzymes for gonadal steroids have been found in cerebral blood vessels (Gonzales et al., 2007). Several studies showing that primary male Human brain microvascular ECs (Zuloaga et al., 2012) and human aorta (Nakamura et al., 2005) express the steroid receptors

AR, ER α , ER β , as well as enzymes involved in 3 β -diol metabolism, 3 α -HSD, 3 β -HSD, 17 β -HSD, and CYP7B1.

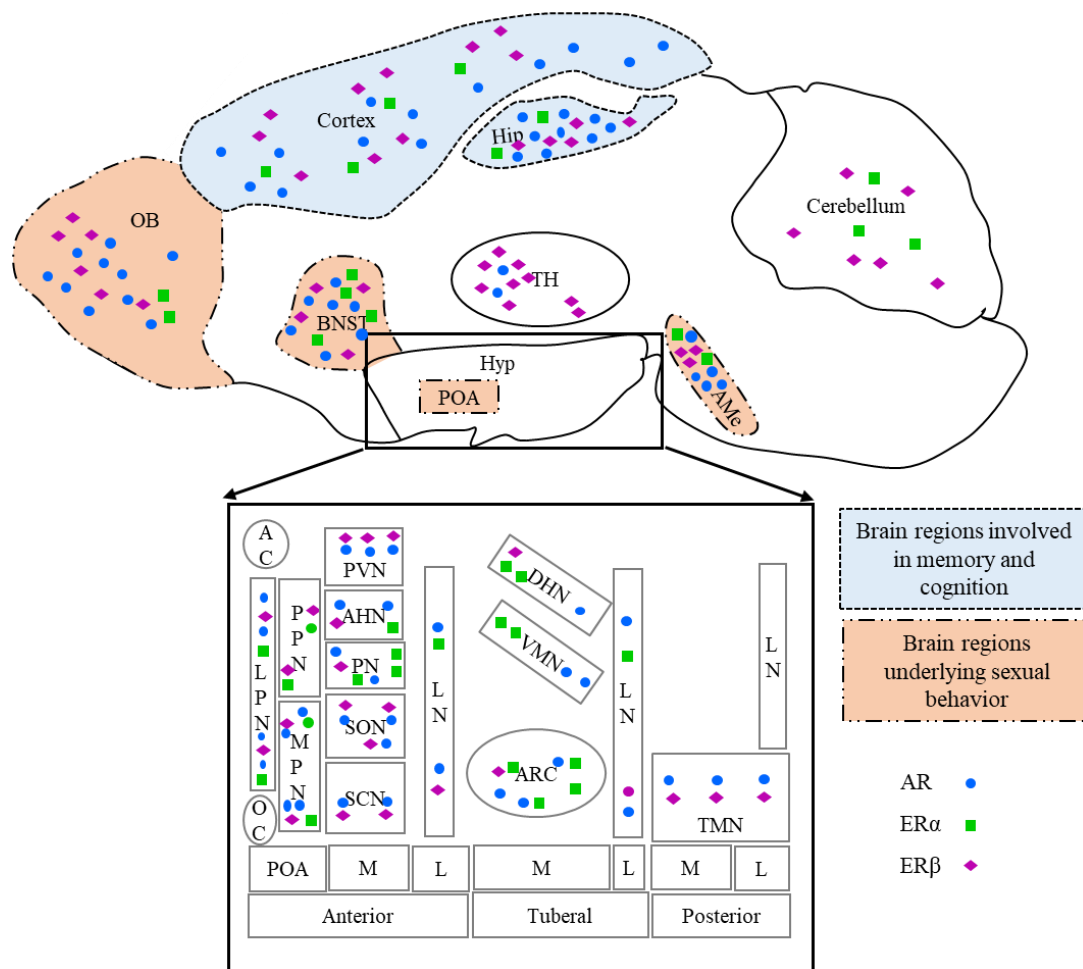


Figure 24. Overview of male brain location and density of sex steroids receptors

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 (Patchev et al., 2004; Shughrue et al., 1997; Simerly et al., 1990). 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, paraventricular preoptic nucleus; PVN, paraventricular hypothalamic nucleus; SCN, supraoptic nucleus; SON, supraoptic nucleus; TH, thalamus; TMN, tuberomammillary nucleus; VMN, ventromedial nucleus (Ahmadpour and Grange-Messent, 2021).

2.4 Structure and function of sex steroid hormone receptors mediating testosterone effects

Sex steroid hormone receptors share a modular structure (**Fig.25**) composed of an unstructured non-conserved N-terminal domain (NTD), a highly conserved central DNA-binding domain (DBD), a moderately conserved carboxyl-terminal ligand-binding domain (LBD) and a flexible hinge region located between the DBD and the LBD (Modi et al., 2016). These domains operate as individual folding units that contribute to the transformation of a transcriptionally active receptor.

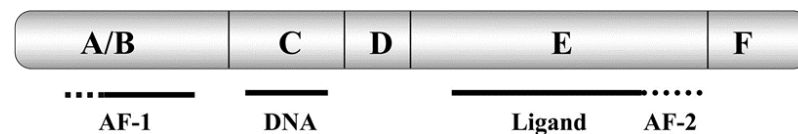


Figure 25. General features of the domain structure of nuclear receptors

The A/B domain at the NH₂ terminus contains the AF-1 site where other transcription factors interact. The C/D domain contains the two-zinc finger structure that binds to DNA, and the E/F domain contains the ligand binding pocket as well as the activation function AF-2 domain that directly contacts coactivator peptides. Adapted from (Nilsson et al., 2001).

Androgen receptor (AR) is a 110 kDa protein including a NTD and the two DBD and LBD which primary structures are well conserved between species (Modi et al., 2016) (Fig.26).

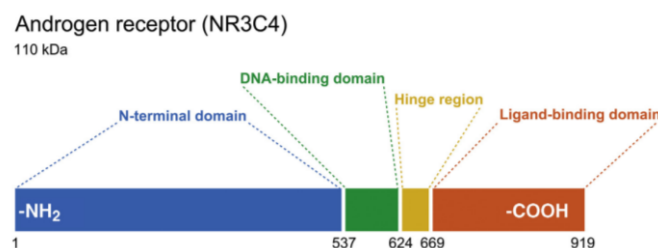


Figure 26. General features of the AR

Figure adapted from (modi et al., 2016)

Unlike the ligand-binding domain and DNA-binding domain which are structured domains, the AR's NTD is intrinsically disordered and mediates protein–protein interactions that are required for transactivation (Leung and Sadar, 2017). In NTD, a trans-activation function named

activation function 1(AF-1)-region, has been established. During AR' transactivation, the AF1 region of the AR's NTD acquires transient folding structures to bind transcriptional coactivators to bridge the AR to basal transcriptional machinery (Bennett et al., 2010). Many proteins have been found to interact with AR. A portion of these proteins was identified as AR co-regulators, either co-activator or co-repressor. AR co-regulators function as transactivation chaperones. They act upon AR with and without bound ligand in a variety of subcellular locations, influencing DNA binding, nuclear translocation, chromatin remodeling, binding interruption of other co-regulators, AR stability, and bridging AR with the basal transcriptional machinery, ligand selectivity and DNA binding capacity (Steketee and Lamberts, 2007). Increased AR co-activator affinity for AR is generally associated with ligand binding and primarily function to enhance AR transactivation. AR co-repressors however, function to inhibit transcription initiation from androgen-responsive genes (Bennett et al., 2010). The number of co-regulators known to interact with AR is considerable and continues to grow. Several great reviews are available with more specific informations on co-regulators (Bennett et al., 2010; Heinlein and Chang, 2002).

The DBD is highly conserved between ER α and ER β with 97% amino acid identity and mediates sequence-specific binding of ERs to DNA sequences in target genes, denoted estrogen-responsive elements. In contrast, the LBDs of ER α and ER β show a 59% overall amino acid sequence identity yet the ligand-binding pockets of the two subtypes show only minor differences in structure. Structural domains of estrogen receptors are presented in (**Fig.27**). Transcriptional activation of ERs is facilitated by two distinct AF, (AF1 and AF2). The NTD contains AF1 region which is responsible for recruitment of co-regulatory proteins. The LBD contains AF2 (Jia et al., 2015).

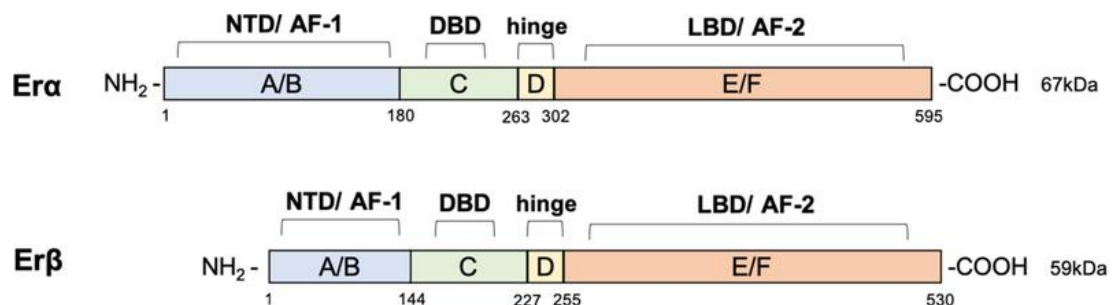


Figure 27. Structural domains of estrogen receptors

Structural domains of estrogen receptor α (ER α) (595aa) and ER β (530aa) are labeled. Both receptors have 6 different structural and functional domains: N- terminal (NTD, A/B domains, AF-1), DNA binding domain (DBD, C domain), the hinge (D domain), the C-terminal region containing the ligand binding domain (LBD, E/F domain, AF-2). Adapted from (Fuentes and Silveyra, 2019).

2.5 Mechanisms of action of sex steroid hormones

ARs and ERs were initially described as having a unique role of transcription factors. However, it is now well established that these receptors exert rapid cellular actions, modulating signaling pathways without translocation in the nucleus. Thus, these receptors mediate signaling pathways modulating several CNS activities by both genomic and non-genomic actions (Bennett et al., 2010; Gonzales et al., 2007; Patchev et al., 2004).

2.5.1 Androgen receptors genomic activity

AR genomic signaling is characterized by slow response indicating a need of DNA binding-dependent actions of the androgens (**Fig.28**).

AR activation proceeds in multiple steps beginning with initial complex formation with certain family of heat shock proteins (also known as chaperonins), binding of ligand, and dimerization. In the absence of androgens, the inactive AR resides in the cytoplasm sequestered by multiple heat shock proteins (HSPs), cytoskeletal proteins and other chaperones. The binding of androgens (testosterone or dihydrotestosterone) triggers the conformational change within the LBD, from the AF2 binding surface then result in AR dissociation from HSPs, thus enabling interaction of AR co-regulators, its dimerization and, finally the receptor complex translocation into the nucleus.

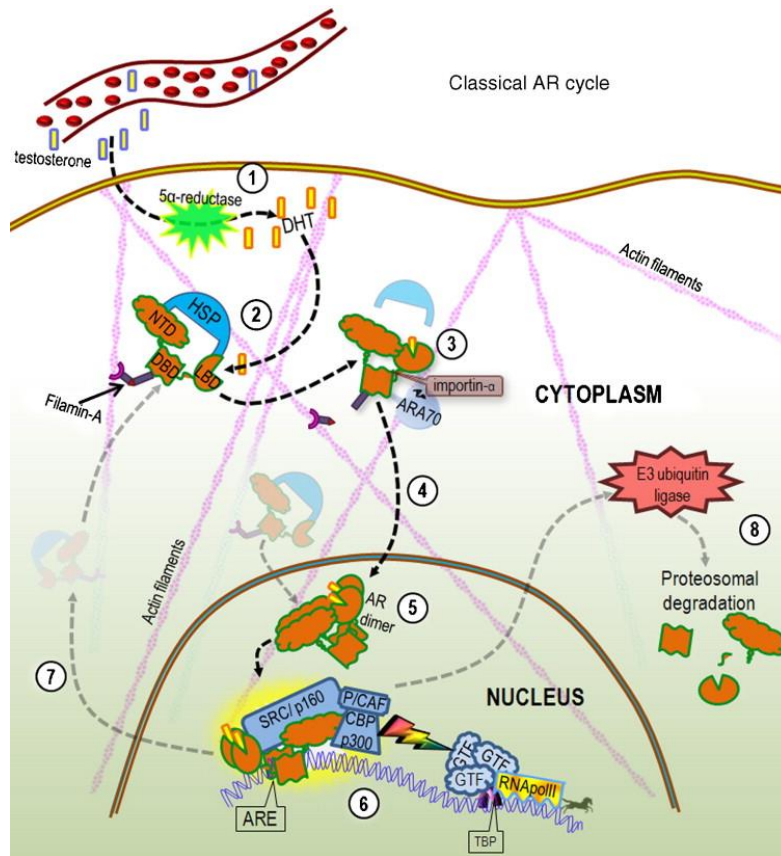


Figure 28. Genomic action of androgen

In the classical pathway, androgen freely passes through the membrane bi-layer and binds cytoplasmic androgen receptor (AR). Bound AR translocates to the nucleus, binds to a DNA response element on a promoter of an androgen responsive gene and stimulates transcription. In more detail Androgen receptor (AR) (coloured orange) is illustrated as a modular protein with each of its domains represented; NTD (N-terminal transactivation domain), DBD (DNA-binding domain), the hinge and the LBD (ligand-binding domain). Following the numbering in the figure: (1) Conversion of testosterone to DHT via the enzyme 5 α -reductase in basal epithelial cells within the prostate; (2) DHT moves into the cytoplasm of epithelial cells where it is bound by AR; (3) Ligand binding to AR induces an AR conformational change whereby proteins such as heat shock proteins (HSPs) dissociate and others such as importin- α and androgen receptor-associated protein-70 (ARA70) are recruited to help stabilise AR and (4) promote AR nuclear translocation; (5) AR dimerises in the nucleus; (6) Numerous other co-activators bind to AR in the nucleus then the AR-DBD facilitates nucleic acid binding at androgen response elements (ARE) which promote recruitment of co-activators with histone acetyltransferase (HAT) activity (CBP/p300, P/CAF), via SRC/p160 co-activator family members, resulting in chromatin remodelling. This allows binding of TATA binding protein (TBP) followed by general transcription factors (GTF) and RNAPolIII to begin transcription; (7) Non-ligand-bound AR is shuttled back to the cytoplasm and re-cycled in preparation for further ligand binding; (8) Alternatively, non-ligand-bound AR can be targeted for proteosomal degradation following ubiquitination by E3 ubiquitin ligase. Adapted from (Bennett et al., 2010).

AR functions most commonly as a homodimer but has been shown to form heterodimers with the orphan nuclear receptor testicular receptor 4 and the ER α isoform, providing variances in transcriptional regulation. The receptor complex translocates into the nucleus and forms

transcriptional co-activator complexes that binds to specific sequences of DNA at the level of hormone response elements (ARE: androgen responsive elements) then recruits transcriptional co-activators as well as the transcription machinery. At last, loss of bound ligand allows the AR recovery to cytoplasm for either preparation for ligand binding or proteosomal degradation. These balance allows regulating AR-protein levels and, thus, genomic activity (Bennett et al., 2010; Brinkmann et al., 1999; Foradori et al., 2008).

2.5.2 Androgen receptors non-genomic activity

AR non-genomic signaling is characterized by speed, with response times being seconds to minutes, indicating a lack of transcription and translation from androgen-responsive genes (**Fig.29**). Concerning the non-genomic manner, androgens interact with a membrane-associated AR (mAR). The mAR is capable to activate G proteins, which results in a rapid and transient increase in intracellular calcium concentration. This increase is mediated either by the activation of voltage-dependent L-type calcium channels (extracellular origin) or by activation of the IP3-DAG signaling pathway which triggers the release of calcium from the endoplasmic reticulum (intracellular origin) (Duval et al., 1983). An increase of intracellular calcium concentration leads to the activation of several signaling pathways via protein kinase C (PKC) or calmodulin (CAM) which activates in particular protein kinase A (PKA) or mitogen-activated protein kinase (MAPK) pathways. Moreover, the DHT' metabolite 3 α -androstenediol has also the ability to activate second messenger pathways by interacts directly with GABA-A receptors. Finally, independent of receptors, channels or second messenger pathways, androgens may mediate some non-genomic actions via their structural properties. Testosterone and DHT have been shown to interact with membrane phospholipids to influence membrane fluidity. Androgen metabolites have been found to acquire additional charges from sulfate residues and, in turn, achieve the necessary charge to penetrate into the lipid/protein complex of the cell membrane, thereby modulate the properties of membrane permeability and excitability of cells (decreasing the membrane flexibility and modulating the actions of enzymes required for ATP hydrolysis) (Aydin et al., 1998; Duval et al., 1983; Foradori et al., 2008) .

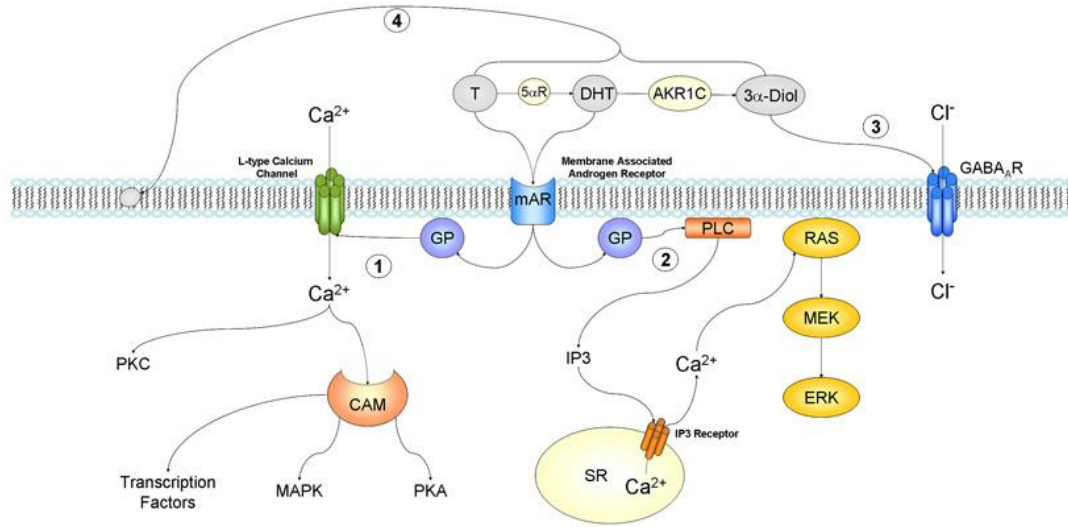


Figure 29. non-genomic action of androgen

Non-genomic androgen actions via changes in intracellular ion concentrations and membrane fluidity. 1) Androgen interacts with a membrane associated androgen receptor (mAR) leading to the activation of L-type calcium channels through some type of inhibitory g-protein (GP). This increase in intracellular calcium can lead to activation of PKC, and via calmodulin (CAM) activate PKA and MAPK pathways, ultimately influencing gene transcription through phosphorylation. 2) Androgen interacts with a membrane associated androgen receptor (mAR) leading to modulation of g-protein activity and subsequent activation of phospholipase C (PLC). Resulting increases in IP3 lead to the release of intracellular calcium stores from the sarcoplasmic reticulum (SR), and consequently the activation of the RAS/MEK/ERK pathway. 3) Dihydrotestosterone (DHT) metabolite 3 α -Diol may interact with the GABA_A receptor and lead to increases in intracellular calcium and thus membrane potential. 4) Testosterone and its metabolites can interact with phospholipids in the membrane bilayer to change membrane flexibility and subsequently alter the function of sodium/potassium ATPase and calcium ATPase. Abbreviations: T = testosterone, DHT = dihydrotestosterone, 5 α R = 5 alpha reductase enzyme, AKR1C = aldo-keto reductase, 3 α -Diol = 3 α -androstenediol, GABA = gamma-aminobutyric acid, GP = g-protein, PKA = protein kinase A, PKC = protein kinase C, CAM = calmodulin, MAPK = mitogen-activated protein kinase, PLC = phospholipase C, IP3 = inositol 1,4,5-triphosphate, SR = sarcoplasmic reticulum, MEK = MAPK/ERK kinase, ERK = extracellular-signal regulated kinase. Adapted from (Foradori et al., 2008).

2.5.3 Estrogen receptors genomic and non-genomic activity

Both physiological and pathological effects of estrogen are mediated by two nuclear ERs: ER α and ER β involved in transcriptional processes (Hamilton et al., 2017), and a number of putative membrane associated estrogen receptors mER involved in non-transcriptional mechanisms (**Fig.30**). The mER may be truncated isoforms of the ER α and ER β receptors such as ER-X and Gq-mER or receptors encoded by genes other than ESR1 and ESR2 such as GPR30 (Micevych and Kelly, 2012). The regulation of transcriptional processes involves nuclear translocation and binding on specific response elements, thus leading to regulation of target gene expression. However, a non-genomic effect involves steroid-induced modulation of cytoplasmic or of cell

membrane-bound regulatory proteins. Indeed, the membrane bound receptor induces cytoplasmic events such as modulation of membrane-based ion channels, second-messenger cascades and transcription factors (Fuentes and Silveyra, 2019). Thus non-genomic mechanisms mediated by estrogens can modify regulatory cascades such as MAPK, P13K, and tyrosine cascade as well as membrane-associated molecules such as ion channels and G-protein-coupled receptors (Pupo et al., 2016).

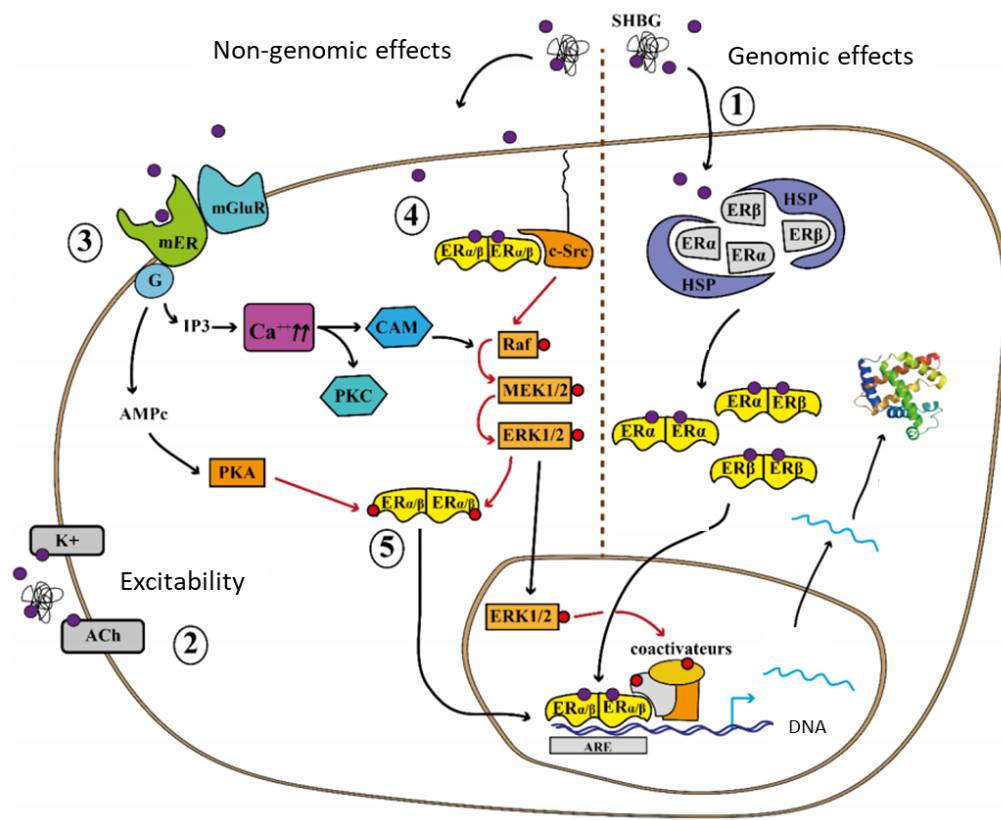


Figure 30. Genomic and non-action of estradiol

Estradiol (purple circles), the majority estrogen, is bound to sex hormone binding globulin (SHBG) in the bloodstream. A free fraction is released near the target cells. The red arrows represent activations induced by phosphorylation. The red circles represent the addition of a phosphate group. (1) The classical route consists of a transcriptional action of ERs, found in two predominant forms ER α and ER β . This action takes several hours to produce visible cellular effects. ERs are found in an inactive state bound to chaperone proteins of the HSP (portal heat shock) family in the cytosol. The binding of estradiol causes the release of ERs which dimerize into homodimers (ER α -ER α or ER β -ER β) or heterodimer (ER α -ER β) then translocates into the nucleus. The active dimers bind to specific sequences (ERE: estrogen response elements) and recruit transcriptional coactivators as well as the transcription machinery. The transcribed mRNAs are then translated in the cytosol which results in the synthesis of new proteins. (2) Estradiol may also act as an allosteric modulator of some K receptors + and certain nicotinic receptors (ACh) which modulate the membrane permeability and excitability properties of cells. (3) Estradiol has the capacity to bind to membrane receptors grouped under the name of mERs coupled to G proteins (membrane associated estrogen

receptor). Activation of mERs results in an increase rapid and transient intracellular calcium concentration through activation of the IP3- signaling pathway DAG or activation of signaling pathways related to PKC, PKA and calmodulin (CAM). Some *mER* also have the ability to interact directly with metabotropic glutamate receptors (mGluR) which potentiates their response. (4) Finally, estradiol can bind to intracellular REs which recruit kinases from the c-Src family, which activates Mitogen-activated protein kinase (MAPK) pathways. (5) Classical and non-classical pathways are interconnected Activation of PKA and ERK1 / 2 can lead to phosphorylation of ERs which induces their translocation into the nucleus without even having bound estradiol. Furthermore, the activated form of ERK1 / 2 (pERK1 / 2) can translocate into the nucleus and phosphorylate coactivators that interact with ERs. Adapted from (Jean and Hardin-Pouzet, 2017).

2.6 Function of sex steroid hormones in the central nervous system

The reproductive effects of sex hormones, estrogens and androgens, are well known. Furthermore, it is increasingly apparent that these hormones have major actions on non-reproductive systems such as the CNS, controlling homeostasis, development and some behaviors for instance (Larson, 2018). These effects can be relevant throughout the lifespan of an organism, and are not limited to the reproductive period (Larson, 2018; Mhaouty-Kodja, 2018a; Picot et al., 2014). Additionally, they have a profound influence on both sexes and not restricted to one sex or the other (Larson, 2018). 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 (Larson, 2018) but the actions of estrogens and androgens often oppose each other (Krause et al., 2011). Moreover, the expression of the respective receptors of estrogens and androgens suggest that cerebral blood vessels are directly targeted by sex steroids and local metabolism of testosterone might balance opposing androgenic and estrogenic influences on cerebrovascular as well as brain function in males (Gonzales et al., 2007). Protective effects of other classes of sex steroids such as progestins, including progesterone, have also been documented (Liu et al., 2010). Numerous therapeutic approaches aim to target the emerging effects of steroid deprivation and/or supplementation for amelioration of several CNS conditions (Gold et al., 2008; Spence and Voskuhl, 2012). Although many studies have investigated the therapeutic potential of estrogens and progesterone in BBB function in females (Spence and Voskuhl, 2012; Voskuhl, 2011), 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.

2.6.1 Non-reproductive function of sex steroid hormones in the female central nervous system

Estrogens exert diverse non-reproductive actions on multiple organs and in multiple physiological systems (**Fig.31**). Specifically, within the CNS, estrogens have profound effects on plasticity and cell survival, neural activity and synaptic plasticity, growth factor regulation, neuroinflammatory cells regulation, apoptosis, cellular proliferation and differentiation (Larson, 2018; Wise, 2002). The neuroprotective efficacy of estrogens has been well described in *in vitro* research including neuronal culture model systems with toxicities including serum-deprivation, β -amyloid-induced toxicity, excitotoxicity, and oxidative stress, as well as *in vivo* investigations in animal models, estrogens have been shown to attenuate neuronal death in rodent models of cerebral ischemia, traumatic injury, and Parkinson's disease (Green and Simpkins, 2000). This protective aspect of estrogens may go some way to explaining the well-known sex differences in the incidence of major cerebrovascular diseases, most notably stroke. Cerebral vessels express ER in both the smooth muscle and endothelial cell layers of cerebral blood vessels (Duckles and Krause, 2007). The aromatase enzyme which is responsible for estradiol synthesis from testosterone, is also expressed within the brain by multiple cell types, including neurons, astrocytes, smooth muscle cells, and endothelial cells. Cerebrovascular endothelial aromatase also plays an important role in cerebrovascular function in female. It has been suggested that the sex differences in cerebrovascular endothelial function in male and female are mediated partly by aromatase. Indeed, an aromatase gene deletion eliminates the sex difference in cerebrovascular endothelial function (Zuloaga et al., 2014).

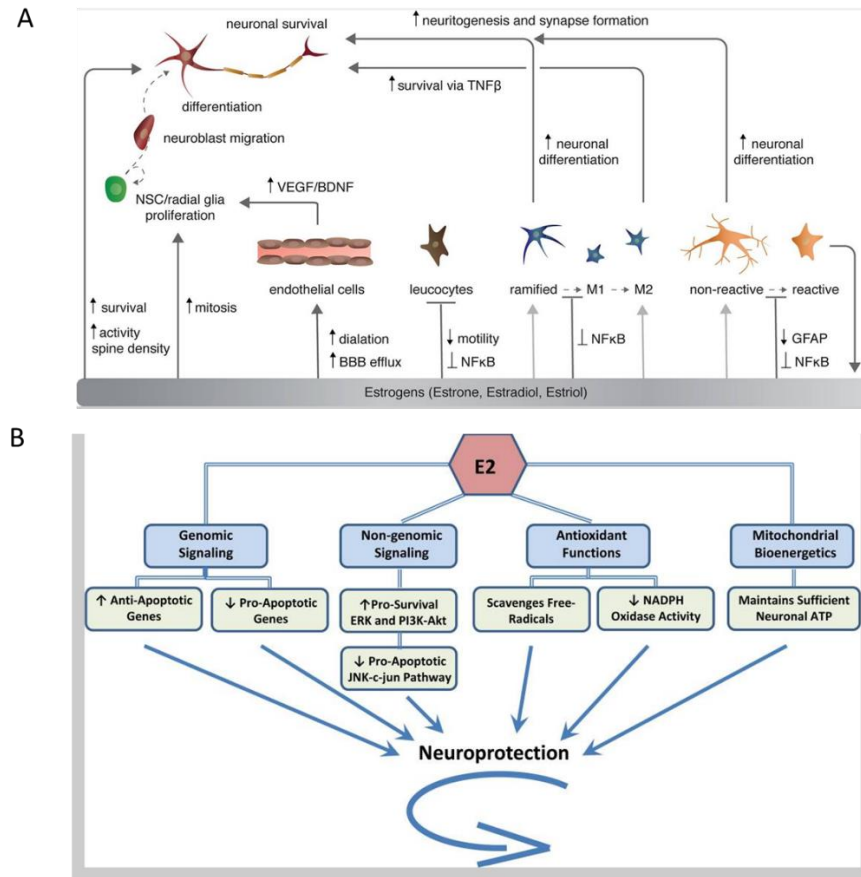


Figure 31. The effects of estrogen on various cell types in the central nervous system

The effects of estrogen on various cell types in the female central nervous system. Light gray arrows indicate that estrogens have an effect on neurons via the given cell type to which the arrow points. Adopted from (Larson, 2018). B) Mechanisms of estradiol neuroprotection. This figure summarizes four major mechanisms through which 17 β -estradiol (E2) protects neurons: classical genomic signaling, rapid non-genomic signaling, antioxidant functions, and mitochondrial bioenergetics. Adopted from (Scott et al., 2012).

Estrogens have been shown to cause vasodilation in cerebral vessels by enhancing eNOS activity and protein levels, and subsequently increasing production of the potent vasodilator NO (McNeill et al., 2002).

Testosterone has the opposite effect in the female, causing an increase in tone by increasing the production of endothelial-derived vasoconstrictors. In addition, it was suggested that estrogen suppresses cerebrovascular inflammation by reducing the induction of inflammatory mediators. In contrast, testosterone augments the cerebrovascular inflammatory

response. Regarding the effects estrogen on the mitochondrial function, it was shown that estrogen modulates mitochondrial function by increasing the efficiency of energy production and by decreasing the production of ROS (Duckles and Krause, 2007) (**Fig.32 A**). It was shown that local production of 17 β -estradiol from testosterone via endothelial aromatase is a reaction that shifts the balance from androgenic to estrogenic effects on cerebrovascular system (Krause et al., 2011). It was hypothesized that aromatase may constitute a negative feedback loop, attenuating and limiting the influence of androgens on vessels by stimulating the vasoprotective effects of locally produced estrogen (**Fig.32 B**) (Krause et al., 2006; Nierwińska et al., 2019).

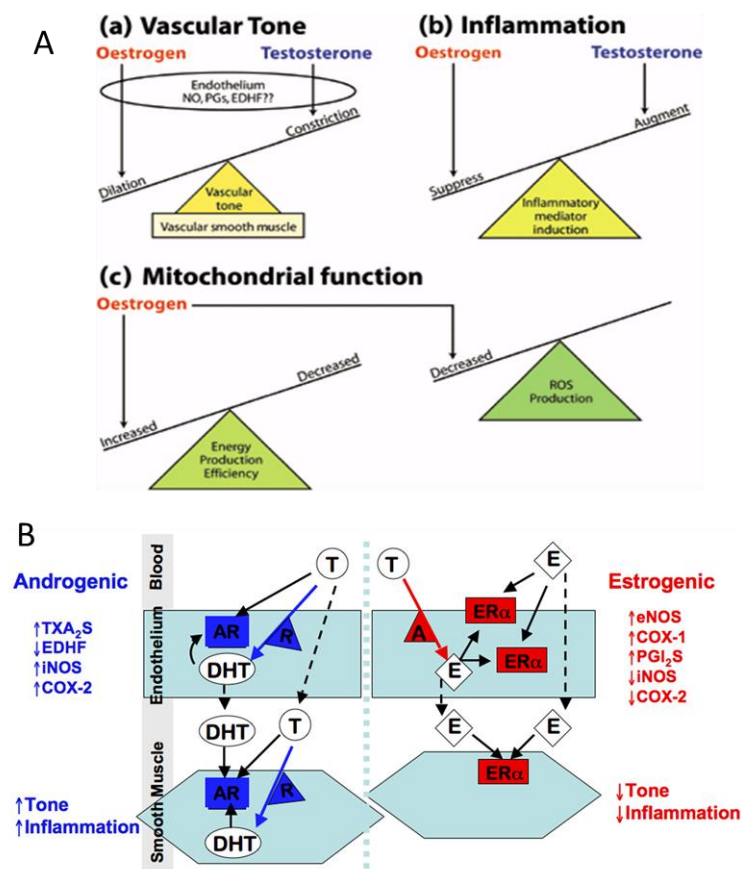


Figure 32. Schematic diagram illustrating local action and metabolism of sex steroids in female cerebral blood vessels

A: Schematic diagram illustrating three major effects of estrogen on cerebrovascular function. Adapted from, (Duckles and Krause, 2007). B: Local action and metabolism of sex steroids in cerebral blood vessels. Adapted from (Krause et al., 2011). It appears that cerebrovascular ARs are stimulated by circulating androgens as well as by local production of the potent metabolite DHT from testosterone, NO, nitric oxide; PGs, prostaglandins; EDHF, endothelium-derived hyperpolarizing factor. A: aromatase; AR: androgen receptor; COX: cyclooxygenase; DHT: dihydrotestosterone; E: estrogen; EDHF: endothelial-derived hyperpolarizing factor; eNOS: endothelial nitric oxide

synthase; ER: estrogen receptor; iNOS: inducible nitric oxide synthase; PGI₂S: prostacyclin synthase; R: 5 α -reductase II; T: testosterone; TXA₂S: thromboxane synthase.

Endothelial cells of the BBB are targets for estrogens. It has been shown that in experimental autoimmune encephalomyelitis (the animal model of multiple sclerosis), estrogens are involved in the regulation of expression of adhesion molecules genes on endothelial cells and are useful for the treatment of experimental autoimmune encephalomyelitis (Dietrich, 2004). Adhesion molecules are involved in the leukocyte recruitment, trafficking, and propagation of neuroinflammation (Alvarez et al., 2011). The use of nanomolar concentrations of 17 β -estradiol has been shown to rapidly, in non-genomic manner, reduced BCRP transport activity in an *ex vivo* BBB model as isolated brain capillaries from male and female rats and mice. Interestingly, removing E2 after 1 h fully restored the BCRP activity which confirms a reversible loss of BCRP transport activity by 17 β -estradiol administration. Consistent with this, estrogen receptors agonists and antagonists, and ER-knockout mice strongly suggest that both ERs are involved in the rapid (minutes) E2-mediated down-regulation of BCRP transport activity in brain capillaries (Hartz et al., 2010).

It has been suggested that estradiol affects ion transporters of BBB. Estradiol could play a beneficial role in ischemia-induced brain edema by reduction of Na–K–Cl transport from the blood into the brain. Estradiol has been shown to attenuate the endothelial cells Na–K–Cl exchange activity of bovine cerebral microvascular endothelial cells in ischemic stroke modeling (Chang et al., 2008; Lam et al., 2009). In ovariectomized female rat, an association between estrogen administration and AQP4 water channel functioning, has been shown. Estradiol treatment highly attenuates LPS-induced upregulation of AQP4 mRNA in the ventral mesencephalon in ovariectomized animals in early and long-lasting induction of AQP4 in perivascular astrocytes (Tomás-Camardiel et al., 2005). The effects of estradiol on ischemic factor-induced astrocyte swelling have been shown in astrocytes cells isolated from rat cortex by both acute and chronic treatment of estradiol. Two or three hours estradiol treatment decreased astrocyte swelling induced by moderate hypoxia and 7 days estradiol treatment reduced astrocytic AQP4 abundance (Rutkowski et al., 2011).

In addition, the impact of estrogens on other membrane transporter of the BBB has been shown. β -estradiol treatment in female rhesus monkeys induced two- to four-fold increases in Glut3 and Glut4 mRNA levels, and lesser but significant, increases in Glut3 and 4 protein levels. E2 treatment induced an ~70% increase in parenchymal Glut1 mRNA levels, but did not appreciably affect vascular Glut1 gene expression. Gluts 3 and 4 were concentrated in cortical neurons and Glut1 in capillaries and glial cells. This result confirms that estrogen induces a rapid response changes in glucose uptake into the brain (Cheng et al., 2001).

A number of studies have provided evidence that estrogens play a pivotal role in blood-brain barrier integrity (Burek et al., 2010, 2014; Shin et al., 2015; Suzuki et al., 2006). Treatment with E2 of both vascular beds including murine endothelial cell lines of brain and heart, led to an increase in trans-endothelial electric resistance and an increase of claudin-5 mRNA and protein levels (Burek et al., 2010). Interestingly sequence analysis have revealed a putative estrogen response element and a putative Sp1 transcription factor binding site in the mouse claudin-5 promoter (Burek et al., 2014).

A study has demonstrated that in female mice, estrogens protect the BBB through enhancement of inter-endothelial cell tight junction and reduction of lymphocyte extravasation after challenge with inflammatory cytokines by down-modulation of ICAM-1 expression. These two actions appear to be governed by different estrogen receptors, with GPR30 regulating paracellular permeability through an increase of the stabilization of tight junctions via modulation of the actin cytoskeleton and ER β limiting lymphocyte migration through suppression of ICAM-1 expression (Maggioli et al., 2016). Elsewhere, it was suggested that in female rats that have undergone transient middle cerebral artery (MCA) occlusion, E2 treatment produced strong protective effects by reduces NF κ B activation, as well as neuronal apoptosis resulting in reducing infarct volume, neuronal apoptosis, and inflammatory responses (Wen et al., 2004). This finding was supported by study of Stice and Knowlton showing that an interaction between E2 and the transcription factor NF κ B has been identified (Stice and Knowlton, 2008). E2 is capable to modulate activity of NF κ B via both genomic and nongenomic actions. E2 can activate NF κ B rapidly via nongenomic pathways, increasing cellular resistance to injury, and inducing expression of the

protective class of proteins, heat shock proteins (HSPs). HSPs can bind to many of the pro-apoptotic and pro-inflammatory targets of NFκB and, thus, indirectly inhibit many of its deleterious effects. In addition, HSPs can block NFκB activation and binding directly. Similarly, genomic E2 signaling can inhibit NFκB, but does so through alternative mechanisms (Stice and Knowlton, 2008).

In a focal cerebral ischaemia model, NFκB signalling contributes to ischaemic brain damage. NFκB is involved in the induction of proinflammatory signaling pathway including cytokines, chemokines, and adhesion molecules (Lawrence, 2009; Ridder and Schwaninger, 2009). In cultured cell line derived from human umbilical vein endothelial cells E₂ was capable to decreases monocytes adhesion, cell adhesion to vascular endothelial cells by inhibition of NF-κB-mediated up-regulation of VCAM-1 expression (Mori et al., 2004). In addition , E₂ has been shown to attenuate astrocyte-derived release of NFκB, IL-1β, TNFα, and MMP-9 in models of inflammatory injury (Witt and Sandoval, 2014). In order to obtain further information on that effect of estrogens on inflammatory response (Pozzi et al., 2006; Witt and Sandoval, 2014).

The influence of reproductive hormones on cerebral blood flow and sex differences in the ability of the cerebral vasculature to increase its blood flow (cerebrovascular reactivity) are relatively new areas of investigation. for more information on cerebrovascular responsiveness in young and older men and women, the readers are referred to a great review in this context (Barnes and Charkoudian, 2021).

2.6.2 Non-reproductive function of sex steroid hormones in the male central nervous system

Although many studies have investigated the therapeutic potential of estrogens and progesterone in females (Spence and Voskuhl, 2012; Voskuhl, 2011), much less attention has been devoted to clarify the impact of estrogens and androgens in males. Therefore, less 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. Thus, the effects of sex steroids on the cerebrovascular system appears to depend on hormones either present in circulation or synthesized locally. Due to the ambiguity of the local metabolism of testosterone, the final effect on the CNS vasculature may be the result of the activity of both testosterone and the more potent DHT as well as estrogen (Krause et al., 2006; Nierwińska et al., 2019).

2.6.2.1 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 (Bielecki et al., 2016; MacLusky et al., 2006).

The decreased susceptibility of men, compared to women, to inflammatory autoimmune disorders such as 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 (Larson, 2018; Mahmoud et al., 2016; Spence and Voskuhl, 2012; Vodo et al., 2013). Furthermore, recent data obtained in a mouse model of chronic demyelination (Hussain et al., 2013) and lysolecithin-induced demyelination (Bielecki et al., 2016) 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 (Forner et al., 2017). 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 (Frye et al., 2004; Jia et al., 2016). Furthermore, the expression of ARs, not only in nuclei (Brown et al., 1995) but also at extra-nuclear sites of hippocampal neurons (Tabori et al., 2005), 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 (Leranth et al., 2003). Replacement therapy by added DHT or testosterone restores spinogenesis through AR signaling, which rapidly promotes kinase networks in neurons (Hatanaka et al., 2015). 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 (Xiao and Jordan, 2002). As already mentioned, levels of sex hormones in elderly people decrease with age, and low levels of androgens in older male individuals have been closely linked to the development of cognitive impairment. Androgens and cognitive impairment in older men are greatly revised in reference (Cai and Li, 2020). Indeed, sex steroid are capable to induce gene transcription or activating signaling pathway that result in the induction of different physiological and pathological events of brain plasticity (Camacho-Arroyo et al., 2020).

In many studies concerning psychiatric disorders, women are reported to have a higher incidence of depression than men (Cohen et al., 2006). Aging is also associated with an increased prevalence of depression in both sexes (Galea et al., 2013). 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 (Mahmoud et al., 2016).

Chronic stress is also associated with reduced neurogenesis and expression of synaptic proteins in the CA1 and CA3 regions of the adult hippocampus (McEwen, 1999). The stress response is mediated through the production of glucocorticoids (Salvador et al., 2014) and it is well known that testosterone reduces glucocorticoids release (Galea et al., 2013; Handa et al., 1994).

In men, the relatively high levels of circulating testosterone, compared to women, is correlated with a higher incidence of strokes (Quillinan et al., 2014). It was shown that the high levels of testosterone and DHT, may contribute toward sensitivity to cerebral ischemia in young males (Cheng et al., 2007; Nakano et al., 2010). The emerging experimental studies, however, indicated that the decline of testosterone level in males can be associated with an increased risk

of stroke upon aging (Cheng et al., 2011). These data describe testosterone dose-dependent neuroprotective and neuro-damaging 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 (Ishihara et al., 2016). Recent investigation revealed a neuroprotective effect of DHT in LPS-induced neuroinflammation male mice. They documented that DHT reduces LPS-mediated amyloid- β deposition and neuronal apoptosis caspase-3, Bcl-2, and Bax, attenuating neuronal damage, and ultimately ameliorating cognitive impairment and motor dysfunction (Yang et al., 2020).

In many brain diseases, disorders and traumas, such as MS, PD, traumatic brain injury, or stroke, neuronal damage is associated with an inflammatory response (Stephenson et al., 2018) and a dysfunction of the BBB (Daneman, 2012). These features induce a neurodegeneration, although the neurodegenerative process becomes independent of inflammatory signals, as has been proven in different models of neurological diseases such as MS (Hampton et al., 2013).

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 (Liu et al., 2011; Malkin et al., 2004; Norata et al., 2006a; Vodo et al., 2013). 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 (Barreto et al., 2007). We will devote another part to a detailed discussion of testosterone effects upon neuroinflammation (see below).

2.6.2.2 Testosterone and cerebral blood vessels

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

Angiogenesis is a multistep process involving endothelial cell migration and formation of lumen-containing tubular structures, regulated by the interactions between ECs and perivascular supporting cells. Angiogenesis is not only critical for embryogenesis and developmental stage under physiological conditions, but also essential in many pathological processes such as wound healing and tumorigenesis (Lindberg et al., 2014).

The development of the BBB occurs in several steps, beginning with angiogenesis from preexisting vessels around embryonic day E10 in mice (Obermeier et al., 2013) (**Fig.33 A**). It has been demonstrated that testosterone modulates vasculature function and integrity via several mechanisms that involve vasculature formation, e.g. proliferation, migration, and differentiation (Campelo et al., 2012). The patterns of circulating levels of testosterone in the male mouse from birth to adulthood are illustrated in (**Fig.33 B**) (Lenz and McCarthy, 2010). Several researchers have focused their activities on testosterone impact on angiogenic mechanisms in cardiovascular regeneration and cerebral angiogenesis in adults (Larson, 2018; Lecce et al., 2014; Sieveking et al., 2010). 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 (Liao et al., 2013). It also increases formation of endothelial cell tubes, by stimulating Smad1 phosphorylation.

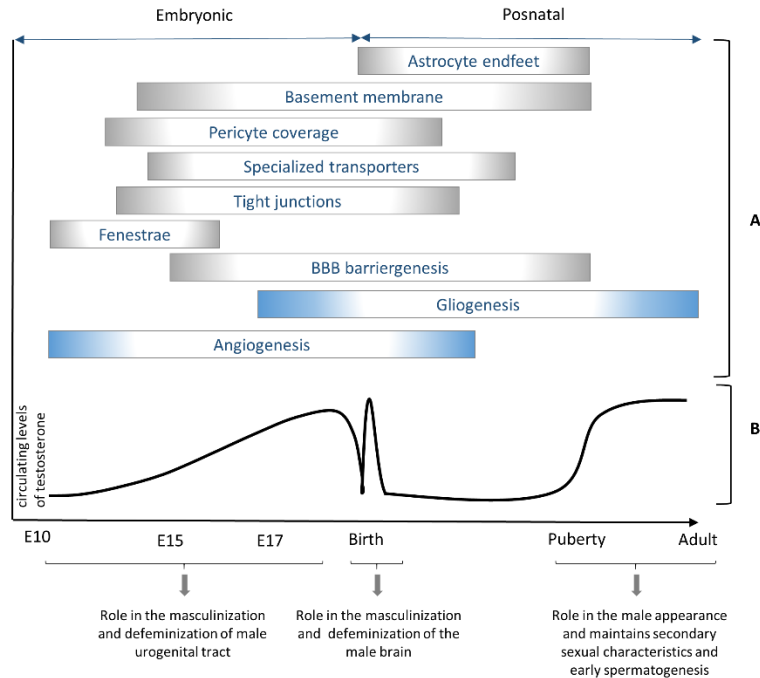


Figure 33. Developmental timeline of the blood-brain barrier set up along with the pattern of circulating level of testosterone in the male mouse

A: Developmental timeline of the blood-brain barrier set up in the murine central nervous system B: pattern of circulating level of testosterone in the male mouse from birth to adulthood E: embryonic days. (Ahmadpour and Grange-Messent, 2021).

This represents one possible mechanism for testosterone-enhanced angiogenesis (Liu et al., 2017a). 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 (Lecce et al., 2014). In addition, VEGF has a therapeutic potential for the induction of neuroprotection, neurogenesis, and cerebral angiogenesis after ischemic disorders such as stroke (Sun et al., 2003). 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) (Chen et al., 2013; Dittrich et al., 2014; Hall and MacDougall-Shackleton, 2012).

The greater incidence of clinical manifestations of heart diseases in men and postmenopausal women compared with premenopausal women reveals a gender differences in

vascular tone and possible vascular protective effects of the female sex hormones. The CBF, in an adult is about 15% (750 mL per minute) of the cardiac output (Ito et al., 2005). Autoregulation of cerebral arteries in response to changes in blood flow and pressure is a well-developed mechanism in the cerebral circulation to maintain constant flow and is critical for maintenance of brain function (Duckles and Krause, 2007). It was found that there is an association between the CBF alteration and CNS dysfunction (Roher et al., 2012). As already mentioned, the cerebral vasculature is a target tissue for sex steroids. Gender differences in vascular tone are mediated at least in part by sex hormone action in the vasculature via specific hormone receptors. Preclinical studies have shown that endogenous and exogenous administration of sex steroids significantly influences both cerebrovascular tone and brain function under normal conditions and following a pathological insult (Gonzales, 2013).

On the one hand no change in vascular contraction in gonadectomized male compared to intact rats, and on the other hands increase in vascular contraction in ovariectomized females compared with intact females, suggest that estrogens are more determinant than androgens (Orshal and Khalil, 2004). There is an intense debate on the role of testosterone on vascular contractility. A number of studies have shown that testosterone facilitates vasoconstriction in rat (*in vivo* and *in vitro*) (Ceballos et al., 1999; Masuda et al., 1991) while nother data have reported the vasodilator effect in rat and rabbit (Costarella et al., 1996; Yue et al., 1995). This could be explained by a vascular function in a concentration-dependent manner with vasodilation in supraphysiological and vasoconstriction in lower concentrations (Honda et al., 1999).

Most studies indicate that exogenous androgens increase vascular tone whereas estrogen treatment decreases these processes by enhancing the NO (Geary et al., 1998) and cerebrovascular prostacyclin synthesis (Ospina et al., 2002). Testosterone and DHT, like estrogen, modulate NO releasing, playing in this way a crucial role in the endothelial contractility (Goglia et al., 2010).

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. 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, basement membrane and TJ proteins of ECs could lead to a modification of microvascular integrity, hence, brain homeostasis. Testosterone modulates cerebral endothelial cells and BBB function through the modulation of key elements of this complex cellular system (**Fig.34**).

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 (Krause et al., 2006; Witt and Sandoval, 2014). 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 the 2017 team 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 pre-optic area (POA) (Atallah et al., 2017), 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 (Atallah et al., 2017). 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 (Willems et al., 2010). 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.

It has been proposed that stress related to physical exercise may impair the functioning of the BBB. Recent investigation on the effect of testosterone in combination with physical training on the blood-spinal cord barrier in adult male Wistar rats has suggested that doses include 8 or 80 mg/kg body weight of testosterone supplementation significantly decreased the levels of occludin, JAM-1, and VE-cadherin TJ proteins but had no effects on ZO-1 or ZO-2 proteins levels (Nierwińska et al., 2019).

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 metabolite (Ohtsuki et al., 2002), is expressed at the BBB (Erdő et al., 2017). The expression of OAT3 in male rats is higher than in females (Kobayashi et al., 2002), and ARs regulate the expression and uptake rate of OAT3 in BBB cells (**Fig. 34**) (Ohtsuki et al., 2005). It is noteworthy that the dopaminergic system participates not only in sexual behavior processes but also in regulation of cognitive behavior (Giuliano and Allard, 2001).

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 BBB (Patching, 2017a). The activity of these transporters is subject to modulation in a variety of neuropathological and neuropsychiatric disorders, including AD, PD (Banks, 2012), and depression (Videbech, 2000). 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 (**Fig.34**) (Desai et al., 2007). It is notable that ovarian steroids are essential modulators of glucose transporters (Patching, 2017b; Shi and Simpkins, 1997). 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 (Pan et al., 2011; Salkeni et al., 2009). 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 (**Fig. 34**) (Miller et al., 2008). P-gp contributes to the efflux of brain-derived amyloid into blood. In addition, TNF α reduces functional activity of BCRP (**Fig. 34**) (Poller et al., 2010), 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 area (Atallah et al., 2017a) although the effects of these shifts upon membrane transporters were not evaluated.

Zuluaga *et al.* have investigated the effects of nonaromatizable androgen, dihydrotestosterone (DHT) and its metabolites, 3 β -diol and α -diol on P-gp expression and reported that DHT and their metabolites reduce the P-glycoprotein expression in male human brain microvascular endothelial cells and their effect on Pgp expression were ER independent (Zuloaga et al., 2012).

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 transduction (Bennett et al., 2010). In addition to brain endothelial cells, the Cav-1 protein has been described in vascular pericytes (Virgintino et al., 2002), while both Cav-1 and Cav-3 have been found *in vivo* and *in vitro* in reactive astrocytes (**Fig. 34**) (Badaut et al., 2015; Virgintino et al., 2002).

It is important to address the controversy concerning the role of Cav-1 in regulating vascular permeability. An *in vivo* study in rats have showed an enhancement of permeability of the BBB through up-regulation of the expression level of Cav-1 (Deng et al., 2011). 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 (Jasmin et al., 2007). 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 death (Jasmin et al., 2007). 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 discussed (Huang et al., 2018).

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 proteins (Nag et al., 2007). 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 inflammation (Stamatovic et al., 2009). Recycling of internalized TJ proteins is crucial for recovery of the brain endothelial barrier integrity (Stamatovic et al., 2009) (**Fig. 34**).

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 (Chen et al., 2018; Stamatovic et al., 2009). 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 B (Bennett et al., 2010). Cav-1 protein is known as ARs co-activator (Bennett et al., 2009, 2010), 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 (**Fig. 34**) (Lu et al., 2001). Given the importance of Cav-1 in modulation of the BBB integrity and its links with ARs, need further investigation in both ARs and their interaction with the Cav-1 protein at the levels of the BBB and their involvement in BBB function is quite clear.

Loss of the basement membrane stability triggered by metalloproteases or collagenases is frequently observed in pathological manifestations involving the BBB breakdown (Fujimura et al., 1999). NO mediates its physiological effects at least in part by the activation of MMPs during matrix reorganization (Ridnour et al., 2007). 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 ECM (pubmeddev and al) and in neuron damage (Candelario-Jalil Eduardo et al., 2011; Lakhan et al., 2013; Rempe et al., 2016). Activated MMPs also lead to BBB leakage by degradation of the TJ proteins (Dhanda and Sandhir, 2018; Rempe et al., 2016; Yang et al., 2016). 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 injury (Gu et al., 2012). 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 (Gu et al., 2012). In a recent investigation, the Cav1/NO/MMP pathway has been suggested as a therapeutic target to ensure protection against cerebral ischemia-reperfusion injury (Chen et al., 2018). 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, located around the cerebral capillaries, share the basement membrane 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 (Berthiaume et al., 2018), there is no data concerning the effects of sex steroid hormones on pericytes. Further investigations in this regard are clearly required.

2.6.2.3 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, glial 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.

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 (Acaz-Fonseca et al., 2016; Barreto et al., 2007; Garcia-Segura and Melcangi, 2006). 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 lesions (Chapouly et al., 2015).

Recent investigation suggests that DHT modulates microglial and astrocyte activation in lipopolysaccharide-induced neuroinflammation male mouse model *in vivo* and *in vitro*. An endogenous DHT depletion by castration exacerbated inflammatory responses in the serum and brain, but these effects were restored by exogenous DHT supplementation (Yang et al., 2020). They have shown that DHT significantly reduced LPS-induced release of proinflammatory factors, including iNOS, NO and TNF α expression levels in BV2 microglial cell lines and decreased the expression levels of COX-2, PGE2 and IL-1 β in LPS-stimulated primary microglia. They also observed that DHT significantly reduced LPS-induced TNF α , IL-1 β , IL-6, iNOS and COX-2 mRNA levels in BV2 cells and primary microglia (Yang et al., 2020). DHT also modulates *in vivo* a microglial and astrocyte activation in LPS-induced neuroinflammation. Castration markedly increased the number of GFAP and Iba-1-reactive cells in the cortex and hippocampus (Atallah et al., 2017).

Interestingly, DHT supplementation in castrated mice restored microglial and astrocyte activation (Yang et al., 2020). Elsewhere, Razmara *et al.* support the notion that testosterone has a profound proinflammatory effect directly on the vascular bed of the brain (Razmara et al., 2005). They have shown that testosterone augments endotoxin-mediated cerebrovascular inflammation in male rat. Chronic *in vivo* testosterone treatment significantly augmented the expression of COX-2 and iNOS in the cerebral vessels of male rats injected with endotoxin. Using four groups of male rats intact, orchiectomized, orchiectomized treated with either testosterone or the testosterone metabolite 17 β -estradiol, they have shown that inflammatory markers was increased in cerebral blood vessels from orchiectomized treated with testosterone rats compared with intact or orchiectomized rats. In contrast, in cerebral blood vessels from orchiectomized treated with 17 β -estradiol rats, there was a significant decrease in endotoxin-induced COX-2 and iNOS protein levels. *In vitro* incubation with LPS (a bacterial endotoxin) also induced COX-2 in pial vessels isolated from the four animal treatment groups, with the greatest induction observed in orchiectomized treated with testosterone vessels compared with the orchiectomized and orchiectomized treated with 17 β -estradiol groups. Production of PGE₂, a principal COX-2-derived prostaglandin end product, was also greatest in cerebral vessels isolated from ORXT rats. (Razmara et al., 2005). LPS is a proinflammatory component of the outer envelope of all gram-negative bacteria that targets the endothelium and directly disrupts EC barrier function *in vitro* and *in vivo* (Bannerman and Goldblum, 1999). It its noteworthy to mention that sex differences exist in immune responses including progression of cytotoxic microglial responses and blood-brain barrier (BBB) disruption to LPS (Cai et al., 2016; Kolmogorova et al., 2021).

These findings were in consistent with previous studies showing that androgen treatment inhibited the expression of NO, TNF α and IL-1 β , as well as increased the synthesis of IL-10 under inflammatory conditions (Malkin et al., 2004). Thus, DHT exerts the anti-inflammatory function by inhibiting microglial activation. Moreover, previous studies have demonstrated that DHT, in human endothelial cells isolated from umbilical cords (Norata et al., 2006b) and testosterone in isolated mouse macrophages (Rettew et al., 2008), downregulates proinflammatory mediators at least in part by decreased the expression of a key trigger for inflammation and innate immunity, Toll-like receptor 4 (TLR4) in microglia at the transcriptional and translational levels. Thus, a

decrease of TLR4 expression on the cell surface could deactivate downstream signaling pathways, responsible for eliciting an innate immune response.

Decrease in the level of DHT in males are reported to be permissive for a chronic inflammatory disease such as MS onset and progression. In EAE Dark Agouti rats (i.e. an experimental model showing a protracted relapsing EAE), DHT exerts a beneficial effect on clinical scores, coupled with decreased gliosis (GFAP) and inflammation (interleukin-1 β , Toll-like receptor 4 and nuclear factor- κ B expression) in the spinal cord, 45 days after EAE induction (Giatti et al., 2015).

Zuluaga *et al.* have examined the effects of DHT and their metabolites, 3 β -diol and α -diol, on cytokine-induced inflammation and reported that they decrease cytokine-induced COX-2 and vascular cell adhesion molecule-1 expression in male human brain microvascular endothelial cells (Zuloaga et al., 2012). They have proposed an alternate pathway for androgen regulation of brain function in which metabolite of DHT induces activation of ER β . The principal metabolite of DHT, 5 α -androstane-3 β , 17 β -diol (3 β -diol), activates estrogen receptor ER β then decreases inflammatory markers in vascular cells (Zuloaga et al., 2012). Their results point to ERs as a potential alternate receptor pathway used by DHT during cytokine-induced inflammation in endothelial cells, because the effects of the DHT metabolite 3 β -diol on COX-2 and VCAM-1 were blocked by ER antagonism.

Furthermore, it was 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 features (Atallah et al., 2017). However it has been proposed that testosterone depletion by castration alone failed to activate microglia or elicit the release of inflammatory factors, suggesting androgen deficiency alone does not induce an inflammatory response in brain but exacerbate preexisting inflammatory conditions (Yang et al., 2020).

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 (da Fonseca et al., 2014). In addition, it has been proposed that in turn, BBB dysfunction contributes to the progression of neurodegenerative diseases (Weiss et al., 2009). 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.

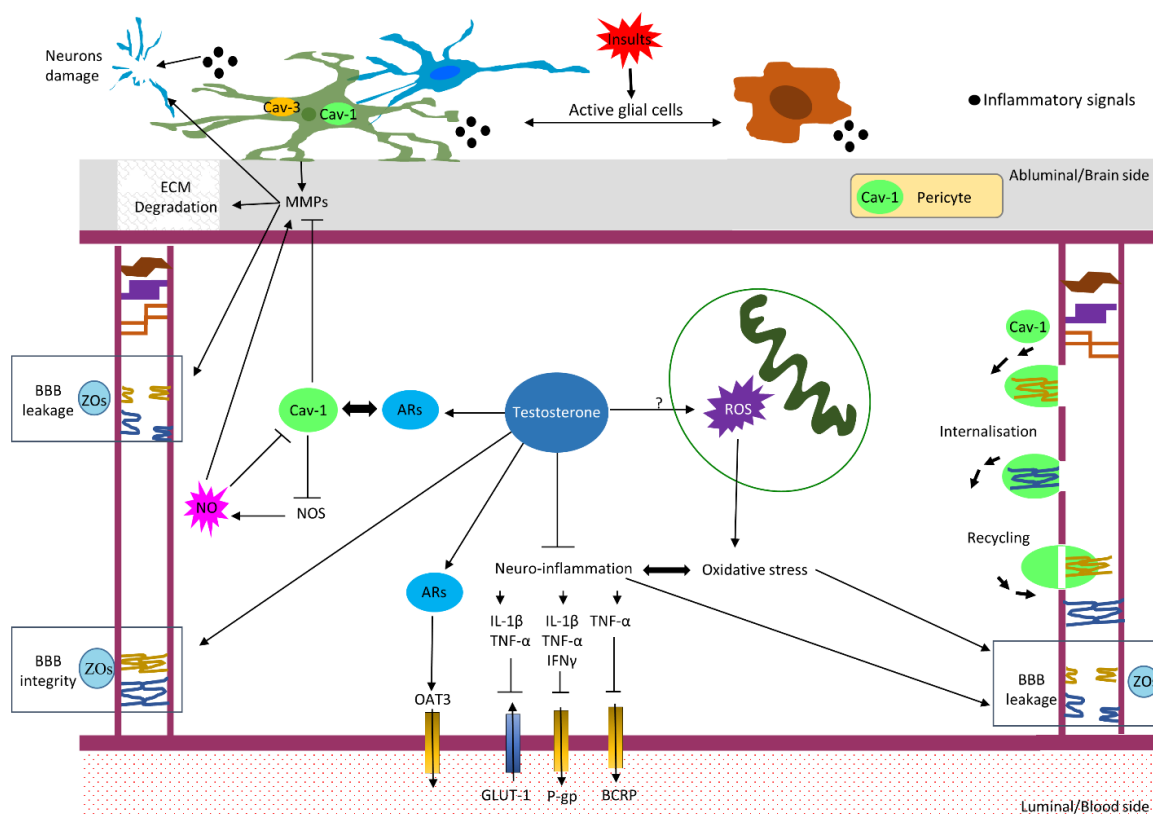


Figure 34. schematic representation of testosterone signaling-induced cellular and molecular interactions in the blood-brain barrier system

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; ZO, zonula occludens proteins. (Ahmadpour and Grange-Messent, 2021).

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

As already mentioned, the sensitivity of the BBB to oxidative stress is due to a high mitochondrial content in cerebral endothelial cells, which is pivotal to maintain active transport and high metabolic work (Oldendorf et al., 1977). Nevertheless, information concerning testosterone impact upon mitochondrial ROS generation in the BBB is still scarce.

Low testosterone levels (Ota et al., 2012; Rovira-Llopis et al., 2017), aging (Liguori et al., 2018), and testosterone-deprivation (Pintana et al., 2015) are risk factors for oxidative stress. It was shown that there is an inverse association between testosterone concentrations and oxidative stress markers in adult men (Haring et al., 2012). 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 homeostasis (Toro-Urrego et al., 2016).

Giatti *et al.* have shown that DHT treatment in EAE Dark Agouti rats (i.e. an experimental model showing a protracted relapsing EAE), the parameters linked to oxidative stress and tissue damage, like thio-barbituric acid-reactive substance levels and Bcl-2-associated X protein expression, and to mitochondrial activity (i.e. content of mitochondrial DNA and proteins) were improved in the spinal cord after DHT administration 45 days after EAE induction (Giatti et al., 2015).

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 (Barron et al., 2006; Cunningham et al., 2014; Grimm et al., 2016; Túnez et al., 2007). Numerous studies have indicated that the effect of testosterone in this regard is tissue, sex and dose dependent (Alonso-Alvarez et al., 2007).

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 (Choudhary et al., 2011). Moreover it was shown that, in these cell lines, testosterone causes the ROS and H₂O₂-induced apoptosis (Tostes et al., 2016). 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 (Kang et al., 2018). However it has been reported that androgens induce ROS generation through AR signaling in cultured vascular smooth muscle (Chignalia et al., 2012) and renal interlobar arteries (Singh et al., 2007).

3 Chapter 3: Sex steroid hormones and environmental factors

The endocrine system is implicated in a range of physiological organism functions through the release of hormones by endocrine glands. These physiologic functions include the control and regulation of all the major functions and processes of the body such as energy control, immunity, behavior, growth and development and reproduction. Chemicals, whether natural or man-made, that interfere with the endocrine system are called endocrine disruptors thereby altering normal hormonal function. the adverse effects of these endocrine-disrupting chemicals (EDCs) on reproductive integrity and function have been widely investigated. Indeed, reproduction disorders in general and decline in male reproductive health in humans and wildlife in particular, is a major concern of the past half century. The reproduction decline, which started in the middle of the 20th century was in parallel with an immense augmentation in the use of natural and synthetic chemicals with endocrine-disrupting activities. It is increasingly evident that environmental factors are a veritable issue in this context and new concerns and other complications have begun and continue to emerge. Fortunately, interest among reproductive biologists has grown rapidly because of the wide range of reproductive effects induced by these chemicals. Thereby the concept of EDCs has risen from total anonymity to become an almost recurrent topic. Although, a lot has been done and environmental issues have become an increasingly important research focus for reproductive biologists but, much more remains to be explored (Hunt et al., 2009).

3.1 The emergence and development of the EDC field

The field of endocrine disruption is in part a scientific subject within the larger context of the environmental movement that appears across the United States in the 1960-1970. Rachel Carson's 1962 book *"Silent Spring"* helped awaken both scientific community and the public to the idea that the production, use and disposal of chemicals as part of human civilization could, and were, inseparable to harm ecosystems and human health. *"The most alarming of all man's assaults upon the environment is the contamination of air, earth, rivers, and sea with dangerous*

and even lethal materials. This pollution is for the most part irrecoverable; the chain of evil it initiates not only in the world that must support life but in living tissues is for the most part irreversible. In this now universal contamination of the environment, chemicals are the sinister and little-recognized partners of radiation in changing the very nature of the world-the very nature of its life” (Carson, 1962). A few years later, the publication of “Silent Spring” , the United States government established the National Institute of Environmental Health Sciences (NIEHS) (1966) to study how the environment affects human health and the Environmental Protection Agency (EPA) (1970) to implement regulations to protect human health and the environment (Schug et al., 2016). The study of EDCs began with the famous meeting at the Wingspread Conference Center, Racine, Wisconsin in July 1991 organized by Theo Colborn and coworkers (Kabir et al., 2015). Figure 35 shows the key events in the research field about EDCs (**Fig.35**).

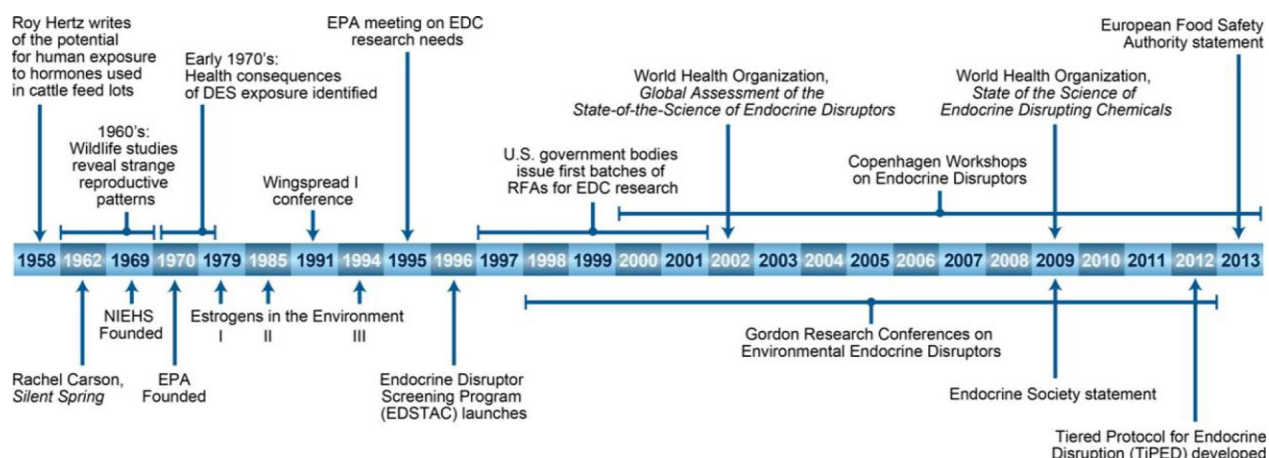


Figure 35. Milestones in the development of the EDC field

Figure adapted from (Schug et al., 2016)

3.2 What are endocrine disruptors?

According to the World Health Organization (WHO), EDCs are exogenous substances or mixtures that cause changes in the functioning of the endocrine system, thus, possibly, causing adverse health effects in exposed organism, its progeny, or (sub) populations (WHO, 2012). In addition to WHO definition, the European Commission also established several criteria in 2017, according to that a substance shall be considered as having endocrine disrupting properties if it meets all of the following criteria (European Commission, 2017): a) It shows an adverse effect in an intact organism or its progeny resulting in a change in the morphology, physiology, growth, development, reproduction or life span of an organism, system or (sub)population that results in impaired functional capacity, impaired ability to compensate for additional stress or increased susceptibility to other influences; b) It has an endocrine mode of action and it alters the function(s) of the endocrine system; and c) The adverse effect is a consequence of the endocrine mode of action. EDCs can be classified according to their nature, origins or their occurrence (**Table 1**) (Kabir et al., 2015). The basis of endocrine system disorders is a set of different possible mechanisms that can lead to disruptions in a normal functioning of the endocrine system such as interference in the processes of synthesis, secretion, transport, metabolism, receptor expression and binding of endogenous hormones (agonist, antagonist) in both wildlife and humans (Czernych et al., 2017).

Table 1. Different type of EDC categories, groups and examples

categories	groups	examples
Nature (Diamanti-Kandarakis et al., 2009)	Natural chemicals	Phytoestrogen: genistein and coumestrol
	Synthetic chemicals	polychlorinated biphenyls (PCBs), polybrominated biphenyls (PBBs), dioxins, Plastics (bisphenol), Plasticizers (phthalates), Pesticides (dichlorodiphenyltrichloroethane (DDT)), Fungicide (vinclozolin) and some pharmaceutical agents (diethylstilbestrol(DES))
Origins (Caliman and Gavrilescu, 2009)	Natural and artificial hormone	fitoestrogens, 3-omega fatty acids, contraceptive pills and thyroid medicines)
	Drugs with hormonal side effects	naproxen, metoprolol and clofibrate
	Industrial and household chemicals	phthalates, alkylphenoletoxilate detergents, fire retardants, plasticizers, solvents, 1,4 dichloro-benzene and PCBs
	Side products of industrial and household processes	polycyclic aromatic hydrocarbons (PAHs), dioxins, pentachlorobenzene
occurrence (Gore et al., 2014)	pesticide	DDT, chlorpyrifos
	Chemicals and food contact materials	Phthalates, bisphenol

3.3 The plastics industry manufactures polymer materials and plasticizers

Man-made or synthetic chemicals like plastics have become an integral part of modern human life (Benjamin et al., 2017). The main polymers that are produced and of importance to our economy, are polyurethane (PUR), polyethylene (PE), polyamide (PA), polyethylene terephthalate (PET), polystyrene (PS), polyvinylchloride (PVC), and polypropylene (PP) (**Fig.36**). (Danso et al., 2019)

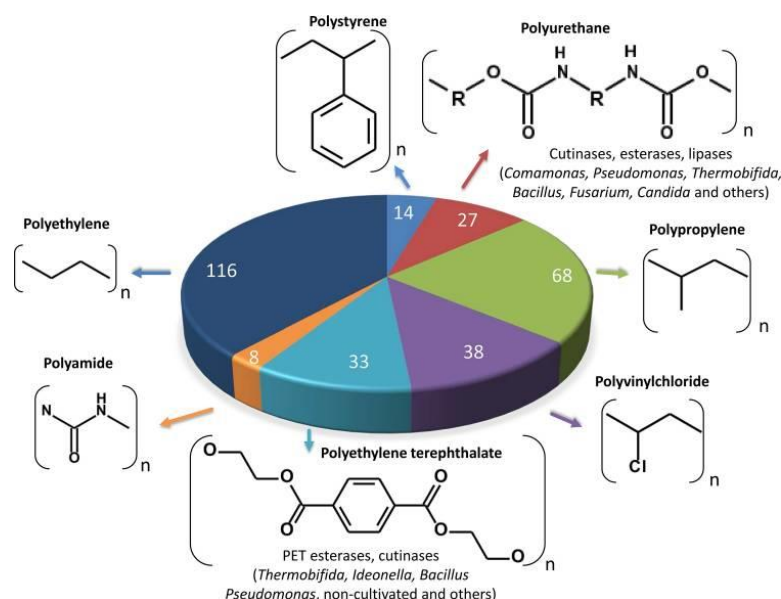


Figure 36. Main synthetic polymers globally produced in 2016

Numbers in the chart indicate the global annual production (millions of tons) of the specified synthetic polymer³. Adapted from (Danso et al., 2019).

Altogether, synthetic polymers are produced worldwide at a scale of at least 350 to 400 million metric tons annually (Danso et al., 2019). Global plastics production has reached 380 million metric tons in 2015, with around 40% used for packaging (Benjamin et al., 2017). It is important to note that plastics' largest market is packaging, an application which growth was accelerated by a global shift from reusable to single-use containers (Danso et al., 2019). Among them, PVC is one of the most widely used plastics, in part due to its low cost, prevalent availability, and tunable mechanical properties (Beveridge et al., 2018). The use of PVC is increasing on a worldwide scale and, along with, it is the use of many compounds added to this polymer to serve a broad variety of functions. Such additives include plasticizers, coloring agents, flow aids, heat stabilizers, and solvents (Erythropel et al., 2014). PVC comes in two basic forms: rigid used in construction for pipe and in profile applications such as doors and windows, and flexible by the addition of plasticizers to polymer. For flexible products composed of PVC, common plasticizers include citrates, aliphatic diesters and phthalates (Beveridge et al., 2018).

3.4 Phthalate plasticizers as one of the largest group of EDCs

Phthalates are compounds obtained by phthalic acid esterification and are used up to 50% (by weight) of PVC products (Genay et al., 2011). Commercially significant plasticizers were discovered shortly after the development of cellulose nitrate in 1846. The production of phthalic acid esters in the 1920 causes a quick shift emphasis away from cellulose nitrate, then was intensified since the commercial availability of PVC in 1931 and the synthesis of di-2-ethylhexyl-phthalate (DEHP) in 1933 (Graham, 1973). Indeed, the application of DEHP as an additive in PVC to impart the flexibility of plastic has made phthalates popular around the world.

The world production of phthalates accounts for more than 18 billion pounds per year, primarily as additives to PVC plastics as plasticizers to impart flexibility, malleability, durability and strength to rigid PVC and as industrial solvents and as components of many consumer products: vinyl flooring, paint, food wrappers, cosmetics, and medical devices (Blount et al., 2000). More than 25 phthalates are used in commercial applications (**Table 2**). Among the EDC compounds, phthalates remain the most frequently detected organic pollutants in the environment worldwide in both urban and rural areas, as recently reported (Gao and Wen, 2016).

Table 2. Common diesters of phthalic acid and their corresponding metabolites studied in the literature

adopted from (Benjamin et al., 2017; Hauser and Calafat, 2005; Staples et al., 1997; Wang et al., 2019)				
IUPAC Name	Common name	Abb.	Major metabolites	Metabolites Abb.
Bis(8-methylnonyl)benzene-1,2-dicarboxylate	Diisodecyl phthalate	DIDP	Mono-(carboxynonyl) phthalate	MCNP
			Mono-(carboxyisononyl) phthalate	MCiNP
Bis(7-methyloctyl)benzene-1,2-dicarboxylate	Diisononyl phthalate	DINP	Mono-isononyl phthalate	MiNP
			Mono-(carboxyisooctyl) phthalate	MCiOP
Bis(2-ethylhexyl)benzene-1,2-dicarboxylate	Dethylhexyl phthalate	DEHP	Mono-2-ethylhexyl phthalate	MEHP
			Mono-(2-ethyl-5-hydroxyhexyl) phthalate	MEHHP

			Mono-(2-ethyl-5-oxohexyl) phthalate	MEOHP (5oxo-MEHP)
			Mono-(2-ethyl-5-carboxypentyl) phthalate	MECPP
			Mono-(2-carboxymethyl-hexyl) phthalate	MCMHP
Di- <i>n</i> -octyl benzene-1,2-dicarboxylate)	Diocetyl phthalate	DnOP/DOP	Mono-(3-carboxypropyl) phthalate	MCPP
			Mono- <i>n</i> -octyl phthalate	MnOP
Di- <i>n</i> -heptyl benzene-1,2-dicarboxylate	Diisoheptyl phthalate	DnHP		
Diphenyl benzene-1,2-dicarboxylate	Diphenyl phthalate	DPhP		
Benzyl butyl benzene-1,2-dicarboxylate	Benzylbutyl phthalate	BBP	Mono-benzyl phthalate (some mono- <i>n</i> -butyl phthalate)	MBzP
Di- <i>n</i> -pentyl benzene-1,2-dicarboxylate	Dipentyl phthalate	DnPP		
Di- <i>n</i> -butyl benzene-1,2-dicarboxylate	Dibutyl phthalate	DnBP	Mono- <i>n</i> -butyl phthalate	MBP
Dipropyl benzene-1,2-dicarboxylate	Dipropyl phthalate	DnPrP		
Diethyl benzene-1,2-dicarboxylate	Diethyl phthalate	DEP	Mono-ethyl phthalate	MEP
Dimethyl benzene-1,2-dicarboxylate	Dimethyl phthalate	DMP	Mono-methyl phthalate	MMP
Phthalic acid	Phthalate	PA		
dicyclohexyl benzene-1,2-dicarboxylate	Dicyclohexyl phthalate	DCHP	Mono-cyclohexyl phthalate	MCHP
bis(2-methylpropyl) benzene-1,2-dicarboxylate	Diisobutyl phthalate	DiBP	Mono-isobutyl phthalate	MiBP

DEHP represents one of the major phthalates in commerce and now, the most abundant in our environment (Wang et al., 2019). In addition to DEHP, other phthalates including diethyl phthalate (DEP), dibutyl phthalate (DBP), butyl benzyl phthalate (BBP), and diisobutyl phthalate (DiBP) are commonly detected in the environment (Gao and Wen, 2016) (**Table 3**). They are currently used in many types of products, including building materials, automotive parts, medical devices, food packaging, cosmetics, perfumes, toys, teethingers, adhesives, paints, floorings,

lubricants, hair sprays, shampoos, soaps, nail polishes, and detergents (Wang et al., 2019). Low molecular weight phthalates such as DEP or DnBP are components of industrial solvents, solvents in perfumes, adhesives, waxes, inks, pharmaceutical products, insecticide materials, and cosmetics. DEP allows perfume fragrances to evaporate more slowly, lengthening the duration of the scent, and a small amount of DnBP gives nail polish a chip-resistant property. Phthalates with longer alkyl chains such as DEHP are used as plasticizers in the polymer industry to improve flexibility, workability, and general handling properties, and 80% of phthalates are used for this purpose (Net et al., 2015).

Table 3. The most often detected phthalates in the environment

Adopted from (Benjamin et al., 2017; Czernych et al., 2017; Dewalque et al., 2014; Martine et al., 2013)	
Abbreviation	Applications
DEHP	Products made of PVC, Medical devices and sanitary products (catheters and tubings (intravenous, nasogastric transfusion, air tubes), containers collection and storage of blood, containers for liquid diffusion, transfusion sets such as blood or dialysis bags, dialysis, equipment, syringe, implants), Construction products and flooring (insulation of pipes, wires, floor tiles and furniture upholstery, wall coverings, cable), Auto parts (upholstery, car seats) Children's products (toys), Food packaging, Solvent in light sticks, plastic films, gloves, and wiring flooring, shower curtains; ethylcellulose, resins (e.g., plastic film, imitation leather, electric wire, cable wearer, sheet, planet, used in nitrocellulose paints., Clothing (parts of shoes, coats against the rain) , <i>etc</i>
BBP	Plasticizer in PVC products such as vinyl floor tiles, adhesives and sealants, car-care products, toys, food packaging, synthetic leather, industrial solvents, personal care products, building and finishing materials (tiles, carpets, foams, sealants, welds, adhesives and varnishes) , <i>etc</i>
DBP	Products made of PVC (widely used as plasticizers in plastics), cellulose materials, personal care products (<i>e.g.</i> , nail polish and solvents, cosmetics), lacquers, varnishes, coatings (<i>e.g.</i> , pharmaceuticals); fsolvents and adhesives <i>etc</i> , excipients in several medications, and scent retainers in certain personal care products (fragrance ingredients in many other cosmetics), cleaning materials, detergents and surfactants, <i>etc</i>
DiBP	Gelling aid in combination with other plasticizers, fiberglass and rubbers, adhesives, paints/lacquers, scent retainer in personal care products (perfume, eye shodaw, moisturizer, mail polish, deodorizer, liquid soap, and hair spray), medication excipients, cellulose plastics and adhesives. <i>etc</i> .
DEP	Personal care products (<i>e.g.</i> , Scent retainer), coatings (<i>e.g.</i> , pharmaceuticals), dyes, insecticides; nail polish as a solvent for nitrocellulose and cellulose acetate, in perfumes as a fixative and solvent, in toilet preparations as an alcohol denaturant; mosquito repellents, camphor substitute, solid rocket propellants, wetting agent, dye application agent, ingredient in aspirin coatings, as a diluents in polysulfide dental impression materials, and in adhesives, plasticizers, surface lubricants in food and pharmaceutical packaging, cosmetics, personal care products cleaning materials, detergents and surfactants <i>etc</i> .

3.5 Phthalate metabolites

In the living organism, phthalate metabolism consists of two parts of biotransformation: hydrolysis and conjugation. By hydrolysis in biologic systems one of the ester groups of the commercial products contain primarily phthalate diesters which are hydrolysed to phthalate monoester metabolite by a process catalysed by lipases and esterases. Then, the metabolites undergo catalysis by enzyme UDP-glucuronosyl-transferase to form the hydrophilic glucuronide followed by its excretion in urine (Ventrice et al., 2013). The most of the toxicity is associated with the phthalate monoester. Through hydrolysis, diesters become more bioactive monoesters with an average half-life of ~12 hr compared to the diester phthalate half-life (< 3 hr) (Hoppin et al., 2002). In addition, microorganisms in water, sediment, soil are able to degrade phthalates. In the environment however, their half-lives depend strongly on natural environment condition such as microbial density and type as well as sunlight irradiation (Net et al., 2015).

DEHP is extensively metabolized after all routes of uptake, and transformed in mono-2-ethylhexyl phthalate (MEHP), which is quickly and extensively metabolized by different oxidation reactions and finally eliminated through urine. Thus, its metabolites are one of the most examined environmental chemicals in human biomonitoring studies (Wang et al., 2019). After 24 hr, 67% of DEHP is eliminated by renal excretion, as some metabolites, including but not limited to MEHP. In the second day, DEHP is eliminated as several metabolites such as 2-carboxy-methyl-hexyl phthalate (MCMHP). Generally, MEHP is considered as a biomarker clearance for DEHP, however MEHP is only a limited metabolite, it represents in fact less than 10% of the original DEHP. The secondary metabolites with a longer half-life could be excellent biomarkers for the evaluation of clearance for DEHP (Ventrice et al., 2013).

3.6 Exposure to phthalates

Phthalates are not chemically but only physically bound to the polymeric matrix, and thus leach, migrate or evaporate into the environment directly or indirectly, during synthesis,

transformation, degradation, application and discard (Heudorf et al., 2007). With time and use, they become ubiquitous environmental contaminants in atmospheric aerosols, sludge from sewage, and wastewater treatment, river and marine waters/sediments, drinking water, biota, and indoor air and atmosphere, foodstuff, other materials, etc (Net et al., 2015). Therefore, consumer products containing phthalates can result in human exposure directly through contact and use, or indirectly through leaching into other products, or general environmental contamination. Humans are exposed through ingestion, inhalation, and dermal exposure during their whole lifetime (Heudorf et al., 2007). Consequently, phthalates and their metabolites have been detected in the human breast milk, blood, and urine (Net et al., 2015). Although the route of DEHP contamination differs by age, lifestyle and occupation but for humans, the potential pathways for exposure to phthalates are contaminated foodstuffs, inhalation, drinking water or dermal contact with cosmetics containing. Nevertheless, food represents the major contributor (more than 67%) of human exposure (Net et al., 2015). The scenario-based risk assessment approach (SceBRA) method has shown detailed information of the exposure to phthalates of european consumers. For instance, it has been proven that infants and toddlers are confronted to higher daily exposures to phthalates (Wormuth et al., 2006). In many cases, exposure can be attributed directly or indirectly to the use of consumer products containing phthalates or releasing phthalates into residences. It has been shown that food is a main source of DEHP, DiBP and DnBP exposure in consumers. In addition, many plasticized consumer products, commodities, and building materials in households lead to a contamination of indoor air and dust. Low molecular weight phthalates such as DMP, DEP, and DnBP are found in the indoor air at high concentrations and high molecular weight phthalates such as DEHP or DINP can be found in house dust in high concentrations (Wormuth et al., 2006). Among EDC compounds, phthalates remain the most frequently detected in most adult individuals tested in industrialized countries (ANSES, 2015; Dewalque et al., 2014; Martine et al., 2013).

3.7 Establishment of reference doses

Reference doses are established from toxicological studies in order to define the safe ingestion dose for human health risk assessments. The dose of NOAEL « non-observable adverse

effect level”, was defined by the WHO in 1990 as the maximum dose of a substance for which no toxic effect is found by experiment or observation in exposed animals during a toxicity study. Once the most appropriate NOAEL value has been established, uncertainty factor (safety factor) which represents a specific area of uncertainty inherent in the available data, was derived to account for: 1) variability in response in the human population, 2) assumed differences in the potency of DEHP between experimental animals and humans, and 3) various other limitations in the database. This factor consists of multiple of 10, a factor of 10 may be introduced to account for the possible differences in responsiveness between humans and animals in prolonged exposure studies (to account for the possibility that humans are more sensitive to the adverse effects of a compound than experimental animals are). A second factor of 10 may be used to account for variation in susceptibility among individuals in the human population (To account for the variability in response between the mean of the healthy population and the response in some proportion of a sensitive subpopulation). Thus, the factor of 100 has been judged to be appropriate for many chemicals including phthalates. The tolerable daily intake dose (TDI) is then determined by dividing the appropriate NOAEL dose by a safety factor and refers to the daily amount of a chemical that has been assessed safe for human being on long-term basis (usually whole lifetime). However, these reference doses are established by typical dose-response curves showing the relationship between a chemical’s concentration and its observed effects. Typical dose-response curves are monotonic, meaning a greater response is observed as the dose increases. However, it is now evident that endocrine disruptors go against predictable or typical dose-response patterns displaying non-monotonic dose responses (Hill et al., 2018) (**Fig. 37**).

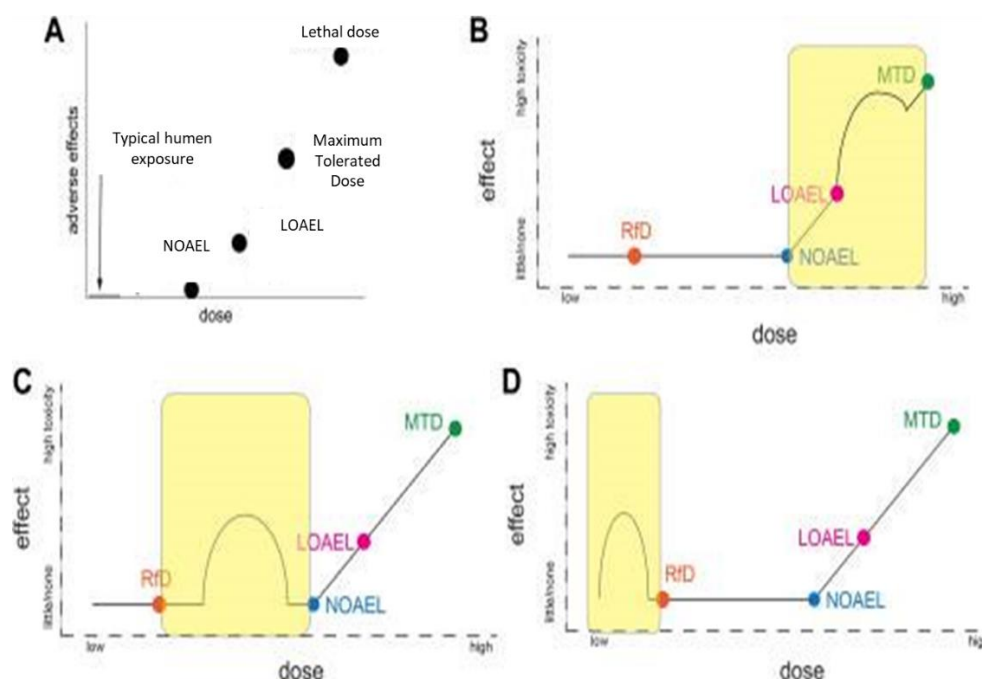


Figure 37. Examples illustrating non-monotonic dose–response curves at different portions of the dose–response curve relative to reference dose

Examples illustrating non-monotonic dose–response curves at different portions of the dose–response curve relative to reference dose. A, Circles indicate doses typically examined in conventional toxicity tests, which are used to calculate the TDI. This figure shows the expected relationship between human exposure levels, the TDI, and the NOAEL. B, Case 1, where non-monotonicity is observed above the NOAEL. C, Case 2, where non-monotonicity occurs at exposures between the reference dose and the NOAEL. D, Case 3, where non-monotonicity occurs at exposures below the reference dose. RfD: reference dose; MTD: maximum tolerable dose; NOAEL non-observable adverse effect level; LOAEL: lowest observed adverse effect level. Adapted from (Hill et al., 2018)

Non-monotonic dose response curves have been demonstrated for natural hormones and EDCs in a variety of biological systems including cultured cells, whole organ cultures, laboratory animals and human populations. (Vandenberg, 2014). With non-monotonic dose responses, the dose-response curve changes direction within the range of doses examined, creating unusual curves, such as “U” or “inverted U” shapes. The mechanisms responsible for these non-monotonic dose response curves are not well known, but it typically relates two principles. The first is the down-regulation of receptors following the presence of the hormone in high concentration, and the second is the presence of two populations of cells that respond differentially in different hormone concentrations (WHO 2012).

- For DBP, a lowest observed adverse effect level (LOAEL) dose of 2 mg DBP/kg body weight (bw) per day for reduced spermatocyte development and effects on the mammary gland was identified from a developmental toxicity study in rats. the European food safety authority (EFSA)'s Panel on contaminants in the Food Chain (ECP) applies to this Point of Departure (PoD) an uncertainty factor of 200 (an extra factor of 2 because of the use of the LOAEL dose instead of the NOAEL dose) for deriving a health-based guidance value (HBGV). The HBGV is a science-based recommendation for the maximum (oral) exposure to a substance that is not expected to result in an appreciable health risk, taking into account current safety data, uncertainties in these data, and the likely duration of consumption.
- For BBP, a NOAEL dose of 50 mg BBP/kg bw per day was identified from a multigeneration study in rats, based on reduced anogenital distance in F1 and F2 males at birth in group exposed to 250 mg/ kg bw per day. The CEP Panel applies to this PoD an uncertainty factor of 100 for deriving a HBGV.
- For DEHP, a NOAEL of 4.8 mg DEHP/kg bw per day based on effects on the testis in F1 animals was identified from a three-generation reproductive toxicity study in rats. The CEP Panel applies to this PoD an uncertainty factor of 100 for deriving a HBGV.
- For DINP and DIDP, EFSA set individual TDIs in its evaluations of 2005 based on liver effects:
- For DINP, a NOAEL of 15 mg DINP/kg bw per day for non-peroxisomal proliferation-related chronic hepatic and renal effects in rats was identified. An uncertainty factor of 100 was applied for deriving the TDI of 0.15 mg/kg bw per day for DINP.
- For DIDP, a NOAEL of 15 mg DIDP/kg bw per day for liver effects in dogs was identified. An uncertainty factor of 100 was applied for deriving the TDI of 0.15 mg/kg bw per day for DIDP (EFSA, 2009, 2011).

It is important to note that, phthalates can cause adverse effects at low doses, below the NOAEL dose, and even below TDI (Hill et al., 2018).

3.8 Concerns related to exposure to phthalates

The main concerns related to exposure to phthalates are the effects on reproduction, in particular in males due to their anti-androgenic action which are demonstrated in human (Jensen et al., 2016), experimental models (Noriega et al., 2009) and wild life (Oehlmann et al., 2009), by *in vivo* and *in vitro* investigations (Radke et al., 2018). These adverse effects include but not limited to fertility problems, reproductive tract development and reproductive behaviors characterized by reduced anogenital distances (reflect alterations in androgen levels or function during the masculinization programming window), decreased male fertility, disturbance of steroidogenesis, DNA damage in sperm, increased urogenital tract abnormality, decrease testicular testosterone levels as well as alteration of sexual behavior (Capela and Mhaouty-Kodja, 2017). An anti-androgenic action of phthalates reflects their effects on a reduction of testosterone production that was observed in prenatal or postnatal exposure to DEHP that affects the pituitary-testis axis and therefore testosterone levels at both low and high doses. However, this axis is not affected by adult exposure neither to high doses ranging from 0.1 mg/kg/d to 20 g/kg/d in rat (Agarwal et al., 1986; Akingbemi et al., 2001) nor to low doses below the TDI dose in mice (Dombret et al., 2017). Referring to the literature, we can realize that the potential peripheral effects of phthalates in particular DEHP on the reproduction are fairly well documented whereas the potential effects on the neural regulation of reproductive behavior and function have received less attention. In addition, most of the studies were carried out at high doses (more than the reference doses NOAEL and TDI or even more than estimated doses for average human exposure). Moreover, despite that the adult brain is under continuous response to environmental stimuli, the majority of studies have been focused on developmental exposure and the outcome of an adult exposure remains unclear and this is an issue that needs serious investigation. Besides the detrimental effects on the reproductive function and behavior, an association between phthalates exposure and CNS function impairment has begun to emerge. The majority of these studies evaluated the effects of prenatal and/or postnatal exposure of DEHP and DBP at high doses on neurobehavioral aspects, with reported changes in aromatase activity, neurotransmitter content or expression levels of synaptic plasticity markers (**Table 4**). The outcome and the

molecular mechanisms underlying adult effects of exposure to low environmental doses of phthalates on neural functions still need to be explored.

Table 4. Effects of phthalates exposure on male CNS

Phthalates and doses	model	Period of exposure/age of analyses	Route of exposure	Effects	CNS regions	Ref.
DEHP at 0, 30, 300, or 750 mg/kg/d	Rat	GD0 to PN21/ PN7, PN14, and PN21	gavage	↓ in the total length and branching numbers of basal dendrites of pyramidal neurons, ↓ of dendritic development related proteins on PN7, PN14, and PN21	Hipp	(You et al., 2018)
DEHP at 0, 10, 50 or 200 mg/kg/d	Mice	GD7 to PN21/pubertal 6-w-old and adult 12-w-old	oral	↓ AR in pubertal (6-w-old), ↓ ERβ (12-w-old); ↑ anxiety- and depression-like status	Hipp	(Xu et al., 2015a)
DINP at 0, 0.2, 2, 20 or 200 mg/kg/d	Mice	6 w-old for 2 weeks/24h after the final gavage	gavage	Histopathological changes in hippocampus (20 and 200 mg/kg/day); ↑ of caspase-3, GFAP, induction of oxidative stress (ROS production) and inflammatory mediators (TNF-α and IL-1β), ↑ of cognitive deficits and anxiety	Cortex and hipp	(Ma et al., 2015)
DEHP at 0 or 10 mg/kg/d	Rat	from PN16 to PN22/ PN26	i.p. injection	↓ Spine density on apical and basal dendrites of dorsal CA3 hippocampal neurons; ↓ BDNF mRNA expression the dorsal hippocampus; Impairment of neuronal and synaptic development in; hippocampus	Hipp	(Smith and Holahan, 2014; Smith et al., 2015)
DEHP at 0 or 10 mg/kg/d	Rat	from PN16 to PN22/ PN26	i.p. injection	↓ axonal markers in the CA3 distal stratum oriens, ↓ cell density of immature neurons in the dentate gyrus (DG) and mature neurons in the CA3; ↓ doublecortin (DCX) in the DG; ↓ synaptophysin in the in the CA3 sub region, adversely impacts on hippocampal development	Hipp	(Smith et al., 2011)
BBP at 0, 50, 250 or 1250 mg/kg/d	Mice	From 4-w-old for 14 days/24h after the final gavage	gavage	↓ 5-hydroxytryptamine (5-HT), ↓ CREB phosphorylation, ↓ glutathione by 250 and 1250, ↑ ROS levels 250 and 1250, ↑ MDA by 1250, impaired learning and memory performance	Hipp	(Min et al., 2014)

DEHP at 0, 1, 10, or 20 mg/kg/d	Rat	From PN16 to PN22/ PN78	i.p. injection	↓microRNAs (miR-132-3p and miR-191-5p) involved in the development and maintenance of hippocampal neurons and dendrite morphology in dose-dependent manner		(Luu et al., 2017)
DEHP at 0, 10 or 750 mg/kg/d	Rat	from GD12 to GD 21/ PN1	gavage	↓Cyclin D1 and Cyclin dependent kinase 1, two critical genes for neuron proliferation by 10 and 750 mg/kg/d; ↑Pro-melanin-concentrating hormone level (involved in feeding behavior, mood and metabolism) by 750 mg/kg/d; deficit of cognitive function at adulthood	Whole brain	(Lin et al., 2015)
DBP at 0 or 500 mg/kg/d	Rat	from GD6 to PN21/ PN21 and PN60	gavage	↑aromase, ↓ER-β, BDNF and p-CREB expression in the hippocampus of immature PND21, no influence on the levels of these proteins in the mature PN60.	Hipp	(Lin et al., 2014)
DBP at 0 or 500 mg/kg/d daily received	Rat	GD6 to PN21/ PN5 PN21	gavage	Neuron loss and structural alternation in neonatal and immature offspring rats (PN5 and PN21); induction of apoptosis (↑number of TUNEL-positive, ↑ caspase-3 activity), ↓synaptophysin, ↓both the slope and amplitude of field excitatory postsynaptic potentials, impaired the spatial learning and memory of offspring rats	Hipp	(Lin et al., 2013)
DBP 0, 25, 75, 225 or 675 mg/kg/d	Rat	from GD6 to PN21 / PN21	gavage	↑ BDNF expression and improves spatial memory in male rats	Hipp	(Lin et al., 2010)
DBP 0, 10 or 50 mg/kg/d)	Mice	From 5-w-old for 2 weeks/7-w-old	i.p. injection	↓In numbers of newly-generated cells in the DG, Disruption of neurogenesis, no effects on neuroinflammatory responses, adverse effects on the hippocampus-dependent learning and memory.	Hipp	(Lee et al., 2019)
DBP at 0 or 500 μM	Cerebella mouse-derived pluripotent neural progenitor cells (NPCs)		24 h incubation	Cell proliferation was reduced by DBP without affecting cell survival; DBP induced G1 phase arrest and increased p53 expression in NPCs, ROS production via mitochondria dysfunction in NPCs; retarded NPC proliferation without affecting cell viability by arresting the cell cycle at G1, ↑DNA damage (↑p53 expression and ↑γH2AX), ↑ ROS levels, mitochondrial dysfunction		(Lee et al., 2019)
DBP (20, 200, 2,000, or 10,000 ppm), DINP (40, 400, 4,000, or	Rat	from GD15 to PN21/ PN7 or PN-week20	food	↑expression of p130 mRNA by 20 and 200 ppm of DBP and all doses of DINP (PN7), ↓expression levels of granulin in by 480 and 12,000 ppm of DEHA. granulin and p130 genes as sex steroid-regulated genes in the neonatal rat hypothalamus that might be involved in sexual	Hyp	(Lee et al., 2006)

20,000 ppm) and DEHA (480, 2,400, or 12,000 ppm)				differentiation of the brain, ↓sexual behavior (decreased copulatory behavior) after maturation (PN-week20)		
Phthalate mixture comprised of 35% DEP, 21% DEHP, 15% DBP, 15% DiNP, 8% DiBP and 5% BBP phthalate. 0, 200, or 1000 µg/kg/d	Rat	through pregnancy and for 10 days while lactating/PN103 and PN134	food	↓cognitive flexibility and a lower number of neurons and synapses in the mPFC in adulthood, ↓in neuron number, synapse number, and size of the mPFC and a deficit in cognitive flexibility.	mPFC	(Kougias et al., 2018)
DEHP or DINP at 0, 0.02, 0.4 or 8 nmol/kg/ week and ovalbumin (allergen) 1 µg /kg/2 weeks).	m Mice	From 6-week old for 7 weeks/ PNW13	gavage	↑mRNA expression levels of the proinflammatory cytokines (IL-1b, TNF-a) the chemokine CCL3, transcription factor NF-kB, microglia marker Iba1 and oxidative stress, by 8 nmol of DEHP in the presence of the allergen, no significant changes were observed, except for reductions in the TNF-a and CCL2 mRNA levels, in mice exposed to DINP combined with the allergen.	Hyp	(Win-Shwe et al., 2013)
DCHP at 0 or 87 nmol/10 µL/animal)	Rat	5-day-old/4 or 8-weeks old	Single intracisternal administration	↓gene expression of the dopamine D4 receptor at 4 weeks old in both the midbrain and the striatum, and the dopamine transporter in the midbrain at 8 weeks old. gene expression of several subtypes of glutamate receptors was facilitated in the striatum at 4 weeks old and in the midbrain at 8 weeks old, abolished immunoreactivity of tyrosine hydroxylase in the substantia nigra at 8 weeks of age, midbrain and the striatum, hyperactivity as a result of degeneration of mesencephalic tyrosine hydroxylase	Midbrain-striatum	(Ishido et al., 2004)
DEHP at 0, 1, 10, or 20 mg/kg/d	Rat	Preadolescent PN16 to 22 /post-adolescent PN78.	i.p. injection	↓tyrosine hydroxylase positive densities in the substantia nigra pars compacta by DEHP (10 and 20 mg/kg)	Substantia nigra	(Holahan et al., 2018)
DEHP 20, 200 µg/kg/d; 500 or 750 mg/kg/d	Mice	from GD10.5 until birth/ F3 PN906100	orally by placing a pipette tip into	↑estrogen receptor in the 20 and 200 µg, and dopamine receptor 1 in the 20 µg/kg/day and 750 mg, anxiety-like behaviors	Amygdala	(Hatcher et al., 2019)

			the cheek pouch			
DEHP at 2, 10 or 50 mg/kg/d	Rat	from GD14 to GD19/ PN0 and PN70	Gavage	Impairment of hypothalamic gene programming and the influences are permanent dependence on developmental stages and nuclei region (Eleven genes were down-regulated in neonatal hypothalamus, 14 genes were altered in adult AVPV, MPN and ARC and most of alterations were found in the 50 mg DEHP group; 50 mg DEHP reduced ER α expression in the ARC, but no alterations were observed in CYP19 expression)	Hyp	(Gao et al., 2018)
DOP 200, 500, and 1000 mg individually or in combination with Pb and As (heavy metals)	Mice	3-week-old treatment exposure once a day for 8 weeks/11 -week-old	Gavage	DOP individually or in combination with heavy metals inhibit the growth and development, impair spatial learning and memory, induce oxidative damage, \uparrow the levels of the caspase-3, Bax and Bax/Bcl-2 ratio, and \downarrow the level of Bcl-2 in the hippocampus of immature mice. A possible mechanism for the neurotoxicity was induced hippocampal neuronal apoptosis in the hippocampus	Hipp	(F eng et al., 2020)
DEHP at 0, 50, and 200 mg/kg/d	Mice	GD7 to PN 21/ PN42	Oral	Impaired spatial learning and memory , \downarrow decreased the levels of the N-methyl-d-aspartic acid (NMDA) receptor	Hipp	(Dai et al., 2015)
DEHP at 0, 3 and 30 mg/kg /d	Rat	since pregnancy onset/PN15	water	\downarrow the inhibitory neurotransmitter GABA and an increase in the stimulatory neurotransmitter aspartate. changes in the hypothalamic contents of the amino acid neurotransmitters, which are involved in the neuroendocrine regulation of reproductive axis	POA	(Carbon e et al., 2012)
DEHP at 0, 200 μ g, 500 mg, or 750 mg/kg/d	Mice	from GD11 until birth / 16-22 months old.	Orally placing a pipette tip into the mouth	\downarrow The number of hippocampal pyramidal neurons, \uparrow of cyclooxygenase-2, 8-hydroxyguanine, and thymidine glycol in their hippocampal neurons, \downarrow AR expression	Hipp	(Baraka t et al., 2018a)
DBP at 500mg/kg	Rat	from GD-6 to PN21/F1, F2, F3	Gavage	The cytoarchitectural alterations (condensed nuclei, vacuole formation and remarkable degeneration, shrinkage of pyramidal neurons in CA1	Hipp	(P and M.j., 2020)

				and CA3 regions; disorganized hilar cells and hyperplasia in dentate gyrus), deficits in cognition and memory		
DEHP at doses of 0.015, 0.045, 0.135, 0.405 and 1.215 mg/kg/d (low doses) and at 5, 15, 45, 135 and 405 mg/kg/d (high doses)	Rat	GD6 to lactation day 21 / PN1 and 22.	gavage	↓ aromatase activity by 0.135 and 0.405mg on PN1 and ↑at 15, 45 and 405 mg.	POA	(Andrade et al., 2006)
DEHP at 0, 30, 300, or 750 mg/kg/d	Rat	from GD0 to PN21/ PN7 and PN 14	gavage	Restrained cerebellar granule cell precursor cellsproliferation and impaired neurodevelopment for males. ↓male pups' protein expressions and mRNA levels of molecules related to proliferation, including Shh, Gli1, N-Myc, CyclinD1 by 300 and 750 mg DEHP. ↓estrogen level and aromatase expression, impairment of neuromotor development.	Cerebellum	(Fu et al., 2019)
DEHP at 0, 0.5, 5 and µg/kg/d	Mice	8-week-old / after 4 wk of exposure	food	No effect on kisspeptin system and circulating testosterone levels, AR downregulation, ↓emission of courtship vocalizations in males, astrocytes activation	POA	(Dombr et al., 2017)

GD: gestation day, PN postnatal day, HIPP: Hippocampus, HYP: hypothalamus, POA: preoptic area, Mpfc: Medial prefrontal cortex

Besides the detrimental effects on the reproductive function and neurobehavioral aspects, an association between EDCs exposure and an impairment of functions of the neurovascular unit (NVU) has begun to emerge. Thus, several studies (**Table 5**) addressed the effects of EDCs exposure on the NVU. The majority of these studies evaluated the effects PCB, with reported changes in

tight junction and the induction of a neuroinflammatory status and oxidative stress. By contrast, only one study addressed the effects of phthalates using high doses of DEHP: 100, 200 and 400 mg/kg/day (Aydemir et al., 2019).

Table 5. Effect of EDCs on the neurovascular unit

Type of endocrine disruptors	model	Mode of administration	Majors effects	References
acute exposure to in an experimental stroke model (subjected to a 40 min ischemia, followed by a 24 h reperfusion) of PCB153 bound to NPs (PCB153-NPs) or PCB1535 at 5 ng/g.	Mice males, 12-week-old	injections through the internal carotid artery (ICA)	elevation of the BBB permeability (increase of sodium fluorescein), disruption of TJ protein (occludin and cl5) expression, increased inflammatory responses VCAM-1, TNF- α IL-1 β , ICAM-1 by both PCB153-NPs and PCB153.	(Zhang et al., 2012)
30 days exposure to PCB153-NPs or PCB153 at the dose of 5 ng/g	Mice males, 12-week-old	gavage	higher PCB153 content in the abdominal adipose tissue and amplified adhesion of leukocytes to the brain endothelium by PCB153-NPs.	(Zhang et al., 2012)
48 h Exposure of ICAM-1 knockout mice to PCB118 at 150 μ mol/kg followed by an injection of Lewis lung carcinoma cells into the carotid artery	4-6 months male mice	gavage	vascular inflammation, overexpression of ICAM-1 and vascular endothelial cell adhesion molecule-1 (VCAM-1) in brain endothelium, induces adhesion and transcapillary migration of tumor cells and, facilitate the development of brain metastases at the BBB level	(Sipos et al., 2012)
30 days by 2 mg/kg/ d PCBs	Rats adult male 90 day old	i.p. injection	Decrease of mRNA expressions of estrogen α and β , Claudin-5, ZO-1 and ZO-2, AF-6 and JAM-3, BDNF signalling molecules (Ras, Raf, MAPK cascade) in hippocampus of PCB treated rats.	(Selvakumar et al., 2018)
30 days by vehicle, Aroclor 1254 at adose of 2 mg/kg/d and a mixture containing almost 70 different PCB isomers	Adult female rats	i.p. injection	Increased oxidative stress markers (H2O2), Lipid peroxidation., Decreased Claudin-5, ZO-1, ZO-2, AF-6 and JAM-3 in cerebellum and hippocampus	(Selvakumar et al., 2013)
acute exposure to coplanar PCB126, mono-ortho-substituted PCB118, and noncoplanar PCB153 (each at 75 or 150 μ mol/kg) and 48h later injected with	14–16 weeks old male mice	oral gavage	increases BBB permeability of sodium fluorescein, Alteration of the TJ claudin-5, and ZO1 in brain capillaries, promote brain metastasis formation in cerebellum, and brain stem	(Seelbach et al., 2010)

luciferase-labeled K1735 M2 melanoma cells into the internal carotid artery				
6h cell incubated with EE2 or BPA each 1 or nM 10	Isolated brain capillaries from Adult Male rats.		EE2 and BPA down-regulated BCRP transport function and expression, BPA had a weak influence. Observed effects by EE2 was reversed by ER-antagonist. BPA effects were not reversed by either antagonist.	(Nickel and Mahringer, 2014)
96 h incubation by toxicant 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) at 10 ⁻¹³ 10 ⁻¹⁰ 10 ⁻⁷ M	three cell types (endothelial cells, brain pericytes and astrocytes) primary cultures of rat (Wistar rat) brain capillary		decrease in trans-endothelial electrical resistance values, the expression ZO-1 and claudin-5 was decreased and low expression of the glial cell line-derived neurotrophic factor (GDNF) mRNA was observed	(Miyazaki et al., 2016)
single dose of Tributyltin-chloride (TBTC) with a 10, 20, and 30 mg/kg and sacrificed on days 3 and 7	Four-week-old male Wistar rats	Gavage	Altered metal homeostasis (iron. Zinc, Calcium) and ROS generation accompanied by elevated lipid peroxidation and protein carbonylation indicated oxidative damage in the striatum than in cerebellum, hippocampus, and hypothalamus.	(Mitra et al., 2015)
1h cell exposure to PCB153 at a concentration range of 1–10 μM	Human brain endothelial cells (hCMEC/D3 cells) derived from human brain microvascular endothelial cells of a female patient		loss of occludin from tight junction assembly, increase of MMP-2 activation, displacement of occludin via the intermediate of lipid rafts	(Eum et al., 2015)
24 h treatment by 5 μM of individual PCB congeners or 10 nM TCDD.	Human brain endothelial cells (hCMEC/D3)		disrupted expression of the cytosolic scaffold proteins of tight junctions, such as ZO-1, ZO-2, and AF6.	(Eum et al., 2008)
28 days exposure by DEHP at 0, 100, 200 and 400 mg/kg/d	6 weeks old prepubertal male rats	oral gavage	Abnormalities in the trace element and mineral levels including Sodium (Na), magnesium (Mg), potassium (K), rubidium (Rb) and iron (Fe) levels in 400 mg/kg/day DEHP treated groups compared to the control	(Aydemir et al., 2019)

3.9 Mechanisms of action of EDCs

In order to analyze the health effects of EDCs, it is necessary to have a clear understanding of their molecular mechanisms of them in cells. By identifying the mechanism of action of EDCs, it is possible to screen out individuals who may be sensitive to exposure to EDCs, or effectively prevent and treat diseases caused by their exposure. The effects of EDCs in several organs are known, but their underlying mechanisms and their mode of action in target cells are not clear. Nevertheless, there are two proposed hypotheses that could explain their effects: the involvement of epigenetic pathways and the mediation through peroxisome proliferator-activated receptors (PPARs), the latter being more related to the effects of phthalates on reproductive function.

3.9.1 Epigenetics

The endocrine system is susceptible to changes in the environment due to its role in maintaining homeostasis. Therefore, the field of epigenetics and the effects on the endocrine system is growing. Epigenetics investigates heritable changes in gene expression occurring without changes in DNA sequence. Disruption of gene expression patterns which are governed by epigenetics can result in autoimmune diseases, cancers, and various other diseases (Zhang et al., 2020). Some environmental factors such as environmental metals (nickel, cadmium, lead, and particularly arsenic), air pollution (particulate matter) and EDCs have been linked to aberrant changes in epigenetic pathways both in experimental and epidemiological studies (Baccarelli and Bollati, 2009). They can modify multiple biological processes such as inflammation and oxidative stress that affect epigenetic mechanisms including DNA methylation, histone modifications, and microRNA expression (Baccarelli and Bollati, 2009). Oxidative DNA damage can interfere with the ability of methyltransferases to interact with DNA thus resulting in a generalized altered methylation of cytosine residues at CpG sites (Turk et al., 1995). Besides these effects, the epigenetic mechanisms are also involved in neuroinflammation from development to neurodegeneration (Neal and Richardson, 2018). Epigenetics is an important mechanism in the ability of environmental chemicals to influence health and disease, and Bisphenol A (BPA) and

phthalates are epigenetically toxic and may play some critical roles in the etiology of many human disease risks. The environmentally induced-epigenetic changes become increasingly relevant to human health and diseases (Singh and Li, 2012).

Available evidence from animal models indicates that exposure to certain EDCs such as Diethylstilbestrol (DES) (Sato et al., 2009) , BPA (Malloy et al., 2019), pesticides (Collotta et al., 2013), persistent organic pollutants (POPs) (Grova et al., 2019), dioxin (Giampaolino et al., 2020), phthalates (Singh and Li, 2012) and the cocktail of different EDCs (Manikkam et al., 2013) during critical periods of mammalian development, may induce persistent and heritable changes of epigenetic states. For instance, it has been shown that DEHP may cause epigenetic changes via DNA methylation and alter gene expression (Singh and Li, 2012).

In the case of the reproductive system, the epigenetic process can regulate genes controlled by nuclear receptors, or genes involved in the control of steroidogenesis, steroid degradation, and reproductive functioning. This regulation controls the degree of hormonal stimulation in the target tissue (Hliseníková et al., 2020).

Fetal testes are a main target for DEHP as evidenced in testicular dysgenesis syndrome due to a reduction in insulin-like hormone 3 (INSL3) expression and testosterone production (Howdeshell et al., 2008). Epigenetic modifications due to exposure to phthalates can lead to various endocrine phenotypes. For instance, maternal exposure to DEHP at a 500 mg/kg/day dose induces an enhancement of DNA methylation levels in testes at gestation day 19 and postnatal days 3 (PND 3). DEHP also significantly increases the expression of DNA methyltransferases and decreases the INSL3 expression until PND 21 (Wu et al., 2010) which may play an important role in abnormal testicular function caused by DEHP. DNA methylation is a process by which methyl groups are added to cytosine or adenine of deoxynucleotides, catalyzed by DNA methyltransferases (Dnmts) (Li and Zhang, 2014). DNA of vertebrate animals can be covalently modified by methylation of the cytosine base in the dinucleotide sequence 5'CpG3' in CpG islands of promoter areas. CpG is an abbreviation for cytosine and guanine separated by a phosphate group, which links the two nucleotides together in the strand of DNA. Molecular and genetic studies in mammals have shown that DNA cytosine methylation (abbreviated to 5mC, for 5-

methyl cytosine) is associated with gene silencing (Li and Zhang, 2014). Indeed, methylation of CpG islands in a gene promoter stably inhibits transcription of that gene. Therefore, DNA methylation is mainly viewed as a major source of genetic silencing in mammalian cells. Both ER α and ER β undergo changes in promoter methylation under physiological condition such as aging (Issa et al., 1994) and pathological conditions including but not limited to breast (Oh et al., 2001) and prostate cancer (Sasaki et al., 2002). Although evidence exists for multiple mechanisms of epigenetic modification of the ER α and ER β genes, DNA methylation has been the most widely described epigenetic phenomenon, and is the first epigenetic mechanism that has been investigated (Wilson and Westberry, 2009). Recent data suggest that an altered ER mRNA expression by BBP might be related to alteration of DNA methylation in the promoter region of ER α (Kang and Lee, 2005).

Recent investigation revealed for the first time interesting associations between urinary levels of phthalate metabolites and expression levels of circulating miRNAs linked to gestational diabetes mellitus (GDM) (Martínez-Ibarra et al., 2019). They observed higher levels of miRNAs (miR-9-5p, miR-29a-3p and miR-330-3p) in patients with GDM compared to non-diabetic subjects with the detection of Phthalate metabolites in 97–100% of urine samples (Martínez-Ibarra et al., 2019). miRNAs are single-stranded, short non-coding RNA sequences (18–24 nucleotides) that regulate gene expression at the posttranscriptional level through base-pairing with complementary sequences of the untranslated regions (UTR) of messenger RNAs (mRNAs), leading to translational repression or mRNA degradation. While majority of miRNAs are detected in cellular microenvironment, surprisingly a handful number of miRNAs, commonly known as circulating miRNA or extracellular miRNA, have also been detected in different biological fluids, including plasma and serum and even in cell culture media. Such extracellular miRNAs are remarkably stable in the extracellular harsh environment with the presence of high RNase activity (Sohel, 2016).

3.9.2 Peroxisome proliferator-activated receptors

Peroxisome proliferator-activated receptors (PPARs) are members of the nuclear receptor superfamily which physiological functions are linked to metabolism, energy homeostasis, cellular

development, and differentiation. Three members of PPARs have been identified, namely PPAR α , PPAR β/δ , and PPAR γ . Despite sharing a high degree of structural homology, the three PPAR isoforms have distinct functional roles, tissue distribution, and ligand-binding properties (Cheng et al., 2019). They play crucial roles in glucose and lipid metabolism, and for atherosclerosis, inflammation and hypertension, the PPARs are considered as important therapeutic targets (Mirza et al., 2019). Several studies carried out using plasmids containing reporter genes for the different PPARs have shown that phthalates have the ability to activate receptors directly (Hurst and Waxman, 2003; Lapinskas et al., 2005). In humans, clinical treatments based on a PPAR agonist induce a decrease in testosterone levels in adults (Vierhapper et al., 2003). The involvement of PPAR receptors in the regulation of metabolism and in the transport of cholesterol, a precursor of the steroid synthesis chain, makes it possible to hypothesize the mechanism of action of phthalates on the decrease in fetal testosterone observed after *in utero* treatment. Taken together, several experimental data introduce PPARs as potential mediators of the effects of phthalates on reproductive function.

3.10 Regulations and restrictions for phthalates

The use of phthalates undergoes the regulations and restrictions due to their potential human and animal health as well as for environmental concerns. The European Union (EU) restricts several phthalates that are considered dangerous for human health under the REACH (Registration, Evaluation, Authorization and Restriction of Chemicals) regulation. But other product-specific legislation also regulates certain phthalates including Cosmetic Products' Regulation (EC/1223/2009), the EU food contact materials framework regulation (EC 935/2004 and Directives 80/590/ECC & 89/109/ECC), Regulation for Plastics Implementation Measure (10/2011/EC), the toy safety directive, and other regulations.

The REACH regulation was adopted in December 2006 by the European Parliament and the council of the European Union to improve the protection of human health and the environment from the risks that can be posed by chemicals, while enhancing the competitiveness of the chemicals industry in the EU. The REACH regulation sets restrictions on any phthalate that is included in the list of carcinogenic, mutagenic or toxic to reproduction (CMR) substances of

category 1A, 1B or 2 under the Classification, Labelling and Packaging (CLP) regulation. Some phthalates such as DEHP, DBP, DiBP, BBP, DnPP, DiPP and DnHP are classified as reproductive toxicants category 1B under Annex VI to the CLP regulation (EC 1272/2008). In addition to their reprotoxic properties, DEHP, DBP, DiBP, BBP and DCHP also have endocrine disrupting properties and have been classified as substances of very high concern (Annex XIV EC 1907/2006) and therefore included in the candidate list for the inclusion in Annex XIV of the REACH regulation (Annex XIV of REACH EC 1907/2006). DEHP, BBzP, DiBP and DnBP are already subject to authorisation (Directive (EU) 2015/863): since February 2015, they must not be used within the European Union without authorisation. The use of phthalate acid esters in toys or objects that can be placed in the mouth of children is now restricted or prohibited in many countries such as UE, U.S., Canada, Argentina, Brazil, and Japan. In addition, for toys and childcare products that might be placed in the mouth, REACH regulation also sets a restriction of 0.1% weight by weight of the plasticized material (individually as a substance or in combination) for the following phthalates: DINP, DIDP, DNOP by entry 52 of Annex XVII to REACH. Since June 2017, three other phthalates are included in the authorisation list: DiPP, DMEP and DnPP.

DEHP was also classified by the EU in 2000 as a priority substance “presenting a significant risk to or via the aquatic environment” in the Water Framework Directive 2000/60/EC, which was updated in 2008 and 2013 (Directive 2013/39/EU of the European Parliament and of the Council of 12 August 2013). EU has proposed a guideline for environmental quality (NQE or Norme de qualité environnementale) of 1.3 µg/L for DEHP in fresh and marine waters. The WHO recommends the concentration of DEHP in drinking water below 8 µg/L (Net et al., 2015). In addition, DEHP appears in the list of priority substances (20 November 2001), priority hazardous substances (12 august 2013) composed by the European Commission to be gradually reduced and definitively eliminated in the long term (Directive 2013/39 / EU, Decree 7/09/2015). Six phtalates including DMP, DEP, DnBP, BBP, DEHP, and DnOP have been included in the list of priority pollutants compiled by both the US and the EU, on the list of priority pollutants in waters (Net et al., 2015). DEHP has been banned for use in cosmetics since 2009 (EC Regulation n ° 1223/2009) and DBP is prohibited in cosmetic products within the EU (Directive 76/768/EEC).

With respect to medical devices in France, the law of 24 December 2012 prohibits the use of compounds containing DEHP in neonatal, pediatric and maternity service in order to reduce the exposure of young children to this substance known for its endocrine properties at a critical period of their life. In addition, all devices containing DBP are to be labelled and the manufacturers should justify its presence in devices intended for use in children, pregnant or nursing women (Directive 2007/47/EC). Furthermore, the directive encourages the replacement of phthalates in medical devices.

4 Thesis Objectives

Phthalates, one of the largest groups of endocrine disruptors chemicals, are largely known as anti-androgenic compounds because exposure to these molecules was reported to reduce testosterone production following a prenatal /postnatal exposure (Capela and Mhaouty-Kodja, 2017). By contrast, pituitary-testis axis is not affected by adult exposure to high doses ranging from 0.1 mg/kg/d to 20 g/kg/d in rat (Agarwal et al., 1986; Akingbemi et al., 2001) or low doses below the TDI dose in mice (Dombret et al., 2017). In the preoptic nucleus, a hypothalamic subregion involved in the expression of the sexual behavior, exposure to low doses of DEHP reduces the number of AR-immunoreactive neurons and AR transcript levels without affecting ER α (Dombret et al., 2017). In addition to well known-role of testosterone in reproductive function and behavior, this sex steroid hormone plays a pivotal role in CNS function including, but not limited to, cerebrovascular integrity (see for review, Ahmadpour and Grange-Messent, 2021). The link between reduced androgen sensitivity, neuroinflammatory process and BBB dysfunction has been demonstrated by our team in the preoptic nucleus of male mice (Atallah et al., 2017). It has been shown that gonadal testosterone chronic depletion in adult male mice, results in a BBB leakage, an activation of astrocytes and microglia, and an up-regulation of inflammatory molecules. In line with these observations, several studies have reported AR-mediated neuroprotective effects of androgens in brain and spinal cord (see for review, Ahmadpour and Grange-Messent, 2021). Thus, NVU function in male is androgen-dependant, it makes the NVU sensitive to exposure to exogenous factors exhibiting some endocrine disruptor properties. Although effects of some phthalate exposures on the central nervous system were investigated, the majority of these studies have evaluated the effects of prenatal and/or postnatal exposure at high doses on neuro-behavior (see table 4). To our knowledge, twenty studies addressed the effects of endocrine disruptors on the NVU, but focusing on polychlorinated biphenyls (PCBs), at high doses (see Table 5). There is only one study which addressed the effects of phthalates, but focusing on high doses of DEHP (100, 200 and 400 mg/kg/d) (Aydemir et al., 2019). However, no analysis concerned the effects of adult exposure to low environmental doses of a phthalate alone or in a mixture on cerebral vessels in male.

Therefore, the overall aim of my thesis was to study the effects of an adult exposure to low doses of DEHP alone or in a relevant environmental phthalate mixture on the neurovascular unit integrity in male.

For this purpose, adult male mice were orally exposed for 6 weeks to DEHP, the most widely detected phthalate in the environment, alone at the tolerable daily intake (TDI) dose of 50 $\mu\text{g/kg/day}$ (DEHP-50 group) or at a 10-fold lower dose (5 $\mu\text{g/kg/day}$, DEHP-5 group), close to the environmental exposure. In order to mimic environmental co-exposure to phthalates, a group of males (Mix group) was exposed to a phthalate mixture containing DEHP at 5 $\mu\text{g/kg/day}$, DBP at 0.5 $\mu\text{g/kg/day}$, BBP at 0.5 $\mu\text{g/kg/day}$, DiBP at 0.5 $\mu\text{g/kg/day}$ and DEP at 0.25 $\mu\text{g/kg/day}$. The composition of the phthalate mixture was based on French and European studies showing an external co-exposure to these molecules and the presence of their metabolites in urinary samples (Anses, 2015; Martine et al., 2013). The ratio of DEHP to the other phthalates was determined on the basis of the estimated daily intake in France and Europe (Dewalque et al., 2014; Martine et al., 2013). Phthalates were incorporated into food and a control group of mice were treated with vehicle-containing food (Vehicle group) (Fig. 38).

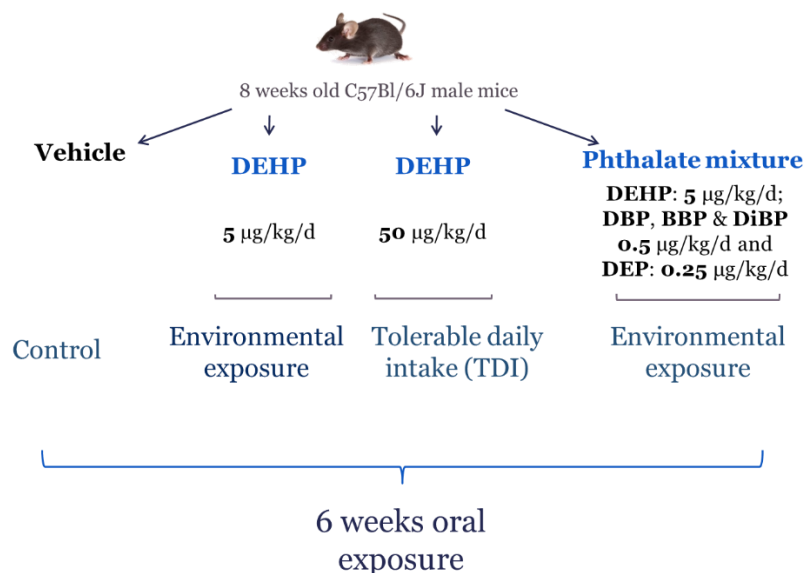


Figure 38. Paradigm of the phthalate exposure

Analysis was focused on two androgen-sensitive brain areas: the hypothalamus medial preoptic area (mPOA) and the three hippocampus subregions CA1, CA3 and dentate gyrus (DG) (Fig.39).

The sex steroid hormone receptors AR and ER α are expressed in the mPOA which is the key region integrating chemosensory signals controlling the male sexual behavior. Male, hippocampus also exhibits a high sensitivity to androgens and expresses AR and ER α (Raskin et al. 2009), which mediate testosterone-dependent modulation of cognitive processes (Picot et al. 2016).

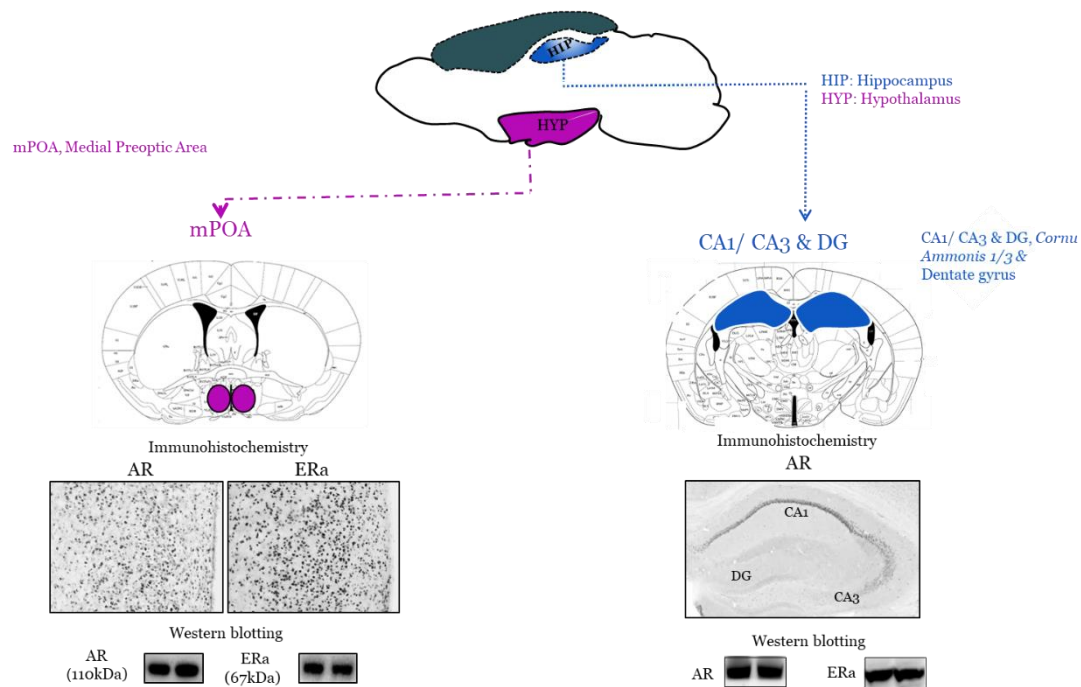


Figure 39. Brain regions of interest

My work thesis is structured with two main objectives that are the subject of the two following parts, part A and part B, of Results section:

The first objective (Part A) was to evaluate if phthalates may impact the cerebrovascular function at doses close to the environmental exposure in adult male mice. My results documented for the first time the effect of oral exposure during adulthood to low doses of DEHP alone or in a phthalate mixture on the integrity of the capillary BBB and surrounding parenchyma in male mice. Data allow to suggest that exposure to endocrine disruptors may be considered as an environmental risk factor for the cerebrovascular function.

Methodology, results and analysis are presented with the corresponding published article (Ahmadpour et al., 2021, Chemosphere; doi: 10.1016/j.chemosphere.2021).

The second objective (Part B) was to bring out an androgen receptor-mediated cellular pathway underlying the hypothalamic mPOA' neurovascular unit impairment previously evidenced, following adult exposure to low doses of di(2-ethylhexyl) phthalate alone or in an environmental phthalate mixture in adult male mice. Data allow to highlight that environmental exposure to endocrine disruptors such as phthalates, could alter AR / Cav-1 interaction, impacting a Cav-1 / nitric oxide / MMP pathway, thus leading to disruption of essential glio-neurovascular coupling to the functioning of neurons. This proposed AR-mediated cellular pathway, would lead to a failure of the glio-neurovascular coupling which could be involved in the phthalate-induced impairment of the male sexual behavior. These data also raise the question of the involvement of anti-androgenic endocrine disruptors in the onset and/or progression of neurodegenerative processes and complications of many brain diseases and injuries.

Methodology, results and analysis are presented with the corresponding article currently submitted for publication.

At last, as the mechanisms underlying the effects of testosterone signaling on the neurovascular unit in males are complex and still poorly studied, I synthesized the data available in the literature in a review article published in *Neuroendocrinology* (Ahmadpour and Grange-Messent, 2021; doi: 10.1159/000509218) (**Annex**). The aim of this review was to summarize and discuss literature, providing insights and contradictions to highlight hypothesis and the need for further investigations. This review presents a summary of the distribution of sex steroid receptors, mediating testosterone effects, in the male brain. Then, it reviews and discuss data concerning the effects of testosterone on cerebral microvessels, focusing on BBB permeability modulation, and also presents an overview of testosterone involvement in neuroinflammation and oxidative stress responses and their relation with BBB alterations.

Results

5 Results

5.1 Part A: Adverse effect characterization of an oral exposure during adulthood to low doses of DEHP alone or in a phthalate mixture on cerebral capillaries and their close environment of testosterone-sensitive brain regions in male mice.

Phthalates are largely known as anti-androgenic compounds because exposure to these molecules was reported to reduce testosterone production. A careful examination of the literature shows that prenatal /postnatal exposure to DEHP affects the pituitary-testis axis and therefore testosterone levels at both low and high doses (Barakat et al., 2018a; Carbone et al., 2012, 2019). By contrast, this axis is not affected by adult exposure to high doses ranging from 0.1 mg/kg/day to 20 g/kg/day in rat (Agarwal et al., 1986; Akingbemi et al., 2001).

Our team has already shown that circuitry is highly vulnerable to adult exposure to low doses of endocrine disruptors (Capela and Mhaouty-Kodja, 2017; Dombret et al. 2017; Picot et al. 2014). In particular, exposure to DEHP at the TDI dose of 50 µg/kg/day or to a 10-fold lower dose, close to environmental exposure, alters sexual behavior by disruption of the emission of ultrasonic vocalizations involved in the ability to attract receptive females, and thus delaying the initiation of mating (Dombret et al., 2017). Proteomic and molecular analyses of the preoptic area showed a down-regulation of the androgen receptor (AR) without affecting ERα (Dombret et al., 2017) which was associated with an alteration of cellular processes such as metabolism, mitochondrial function, protein folding, cellular stress and neuroprotection, transcription or cytoskeleton plasticity (Dombret et al., 2017).

Among other, the link between reduced androgen sensitivity, neuroinflammatory processes and BBB integrity has been recently demonstrated by our team in the preoptic nucleus of male mice (Atallah et al. 2017). The BBB is a physical and metabolic diffusion barrier, which acts as a selective and highly regulated interface between the circulatory system, and thus the immune system, and the brain parenchyma. The BBB comprises tight junctions-sealed brain endothelial cells containing sparse transport vesicles, pericytes, astrocytes end-feet, and basement membrane. Accumulating evidences suggest that BBB function is compromised during

inflammation. The brain endothelium serves as both a source of, and a target for, inflammatory molecules and reactive oxygen species (Erdő et al., 2017).

It was already shown that testosterone chronic depletion in adult male mice by castration inducing neural AR down-regulation, results in activation of astrocytes and microglia and up-regulation of inflammatory molecules (iNOS, COX2, IL-1 β , TNF α). These males also exhibited increased capillary BBB permeability for exogenous tracers and endogenous immunoglobulins, underlined by an altered organization of tight junction ultrastructure, and reduced expression levels of tight junction proteins.

In this context, our work aimed to document for the first time the impact of an oral exposure during adulthood to low doses of DEHP alone or in a phthalate mixture on the integrity of the capillary BBB and surrounding parenchyma in male mice. Analyses focused on two androgen-sensitive brain areas as mentioned above, the hypothalamic mPOA and hippocampal subregions.

It is generally accepted that increased permeability or leakage of the BBB, reflects the failure of its functional integrity. Thus, the BBB leakage induced by phthalate exposure in the hypothalamus mPOA and hippocampus CA1, CA3 and DG sub-regions, was assessed with analysis of the extravasation of an exogenous tracer (4-kDa Evans Blue dye) and endogenous immunoglobulins.

The combination of these two different tracers was performed to overcome the limitation of one given tracer, and their detection in the parenchyma (called extravasation), indicated a BBB leakage. The two major causes of BBB leakage are tight junction disorganization (paracellular diffusion) and enhanced transcytosis through endothelial cells (transcellular diffusion alteration). The organization of tight junction structure was then analyzed in order to determine whether BBB dysfunction was a consequence of the alteration of their structure. Disorganization of tight junction's structure can be caused by changes in the expression level of tight junction proteins. This analysis was then focus on claudin-5, occludin and ZO-1. The amount levels of tight junction

proteins were measured by Western blots performed on microvessel-enriched fractions obtained from hypothalamus and hippocampus.

In order to evaluate the transcellular diffusion, Caveolin-1 (Cav-1), the main component of caveole and main caveolin protein isoform expressed by all the cell types that form the vascular wall of murine BBB (endothelial cells, pericytes and astrocytic end-feet), was investigated. Cellular distribution of Cav-1 was examined by immunohistochemistry, and amount levels was analyzed by Western blot performed on microvessel-enriched fractions obtained from hypothalamus and hippocampus.

The neuroinflammation is often associated with a dysfunction of the neurovascular unit. Neuroinflammation was then studied by analyzing glial activation and expression levels of inflammatory molecules as described in detailed in the article. Briefly, the cellular distribution of Iba-1 (marker of microglial activation), and GFAP, S100 β , NDRG2 (markers of astrocytes activation) as well as COX-2 and iNOS (NO-producing enzyme associated with inflammation) was examined by immunohistochemistry, and their corresponding amount levels were analyzed by Western blot performed on microvessel-enriched fractions from hypothalamus and hippocampus.

Our results showed that subchronic exposure of adult males to environmental doses of phthalates leads to an impairment of the BBB in two cerebral androgen-sensitive areas, the hypothalamus mPOA and the hippocampus CA1 and CA3 sub-regions. This BBB leakage was observed following an exposure to the TDI dose or to a 10-fold lower dose, close to environmental exposure, of DEHP alone or in a relevant mixture containing the most commonly phthalates detected in the environment. Capillaries exhibited a BBB leakage for exogenous tracers and endogenous immunoglobulins which was underlined by an alteration of tight junction organization and caveolae-mediated transcellular transport. In contrast, and interestingly, no BBB leakage was detected in the hippocampal DG whatever the exposure.

This alteration of the BBB is associated with glial activation affecting microglia and/or astrocytes. In the hypothalamus mPOA, activated microglia were characterized by an increase of Iba-1 protein level with the TDI dose of DEHP, and exhibited also a COX-2 increased protein level.

In hippocampal CA1 and CA3 sub-regions, the environmental dose of DEHP (5 µg/kg/day) led to microglial activation without COX-2 expression. Activated astrocytes were only observed in the parenchyma and closed to capillaries of the mPOA with an increase of GFAP, NDRG2 and S100β protein levels, which was also associated with an increase of iNOS protein level in these cells. In the hippocampus, whatever the sub-region and the exposure, no activated astrocytes were detected whereas iNOS protein levels were enhanced in CA1 and DG sub-regions but not co-localized in astrocytes.

This study shows that phthalates induce adverse effects on the cerebrovascular function at doses close to the environmental exposure in adult male mice. Subchronic exposure to low doses of DEHP alone or in a phthalate mixture increases BBB permeability of two brain regions leading to a dysfunctionality of this selective interface, which is needed to ensure a tight regulation between the circulatory system, the immune system and the brain parenchyma, but similarities and differences in the vulnerability of the NVU of those two brain regions were highlighted. This disruption of the neurovascular unit in the mPOA and hippocampus could participate respectively to the reported-sexual alteration and cognitive dysfunction following phthalates exposure.

In conclusion, phthalates, another family of endocrine disruptors, can also impact the cerebrovascular function at doses close to the environmental exposure, thus suggesting that exposure to endocrine disruptors may be considered as an environmental risk factor for the cerebrovascular function. In addition, these analyses show that DEHP-induced adverse effects can be observed at low doses equivalent or below the tolerable daily intake dose of 50 µg/kg/day, which was established and recently updated by the European Food Safety Authority, raising the question about the determination of the TDI dose for phthalates in humans.

Article



Disruption of the blood-brain barrier and its close environment following adult exposure to low doses of di(2-ethylhexyl)phthalate alone or in an environmental phthalate mixture in male mice

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ARTICLE INFO

Handling Editor: Alvine C. Mehinto

Keywords:

Blood-brain barrier
Endocrine disruptors
Phthalates

ABSTRACT

We have previously shown that adult male mice exposure to low doses of di(2-ethylhexyl)phthalate (DEHP) alters neural function and behaviour. Whether such exposure also affects the integrity and function of the blood-brain barrier (BBB) remained to be explored. The impact of adult exposure to low doses of DEHP alone or in an environmental phthalate mixture on the BBB integrity and surrounding parenchyma was studied in male mice. Two-month-old C57BL/6J males were orally exposed for 6 weeks to DEHP alone (5, and 50 µg/kg/day) or to DEHP (5 µg/kg/day) in an environmental phthalate mixture. BBB permeability, glial activation and neuro-inflammation were investigated in the hypothalamic medial preoptic area (mPOA) and hippocampus involved, respectively on the reproductive and cognitive functions. Exposure to DEHP alone or in a phthalate mixture increased BBB permeability and affected the endothelial accessory tight junction protein zona occludens-1 and caveolae protein Cav-1 in the mPOA and the hippocampal CA1 and CA3 areas. This was associated with an inflammatory profile including astrocyte activation accompanied by enhanced expression of inducible nitric oxide synthase in the mPOA, and a microglial activation in the mPOA and the hippocampal CA1 and CA3 areas. The protein levels of the inflammatory molecule cyclooxygenase-2 were increased in activated microglial cells of the exposed mPOA. None of the major effects induced by DEHP alone or in a mixture was detected in the hippocampal dentate gyrus. The data highlight that environmental exposure to endocrine disruptors such as phthalates, could represent a risk factor for the cerebrovascular function.

1. Introduction

Phthalates are chemical compounds found in plasticizers and solvents (Kabir et al., 2015) and are among the most frequently detected organic pollutants in the environment. Di-2-ethylhexyl phthalate (DEHP), the most commonly detected phthalate, is widely used to add flexibility to high-molecular-weight polymers used in the manufacture of polyvinyl chloride plastic and is therefore found in containers for the storage of food and beverages. DEHP was also classified by the EU in 2000 as a priority substance “presenting a significant risk to or via the aquatic environment” in the Water Framework Directive 2000/60/EC, which was updated in 2008 and 2013 (Directive, 2013/39/EU of the European Parliament and of the Council of 12 August 2013). In addition to DEHP, other phthalates including diethyl phthalate (DEP), dibutyl phthalate (DBP), butyl benzyl phthalate (BBP), and diisobutyl phthalate

(DiBP) are also detected in the environment worldwide (Gao and Wen, 2016).

The majority of the *in vivo* studies addressing the effects of phthalate exposure on the nervous system have focused on the effects of perinatal exposure. Furthermore, the effects of exposure to phthalates at low doses during adulthood are still largely under-explored. Our recent study revealed that chronic exposure during adulthood to DEHP at the tolerable daily intake dose (TDI) of 50 µg/kg/day (European Food Safety Authority (EFSA) 2005, 2019) or to a 10-fold lower dose (5 µg/kg/day), close to environmental exposure, disrupts the emission of courtship vocalizations and therefore the initiation of mating in male mice (Dombret et al., 2017). This behavioural alteration was not due to modifications of circulating testosterone levels and/or the integrity of the hypothalamic–pituitary–gonadal axis. It was instead associated with down-regulation of the androgen receptor (AR) in the hypothalamic

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<https://doi.org/10.1016/j.chemosphere.2021.131013>

Received 21 January 2021; Received in revised form 14 April 2021; Accepted 26 May 2021

Available online 31 May 2021

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medial preoptic area (mPOA), the main cerebral area involved in the expression of male sexual behaviour (Dombret et al., 2017). The neural AR plays an important role in the expression of male sexual behaviour (Raskin et al., 2009) and also in the modulation of hippocampal functions as shown by the impaired temporal order memory in mice lacking the neural AR (Picot et al., 2016). The male hippocampus does indeed exhibit high sensitivity to androgens and can thus be targeted by compounds exhibiting anti-androgenic activities. In this context, a previous study showed that prenatal or perinatal exposure to DEHP at 200 µg/kg/d or 10–200 mg/kg/d impaired spatial memory and induced anxiety- and depressive-like behaviour in adult male mice, and that these effects were associated with AR down-regulation in the hippocampus (Barakat et al., 2018; Xu et al., 2015).

In the brain, including the hypothalamus and hippocampus, the blood-brain barrier (BBB) present at the level of the cerebral capillary protects cerebral regions from the toxicity of circulating xenobiotics and pathogens thereby providing cerebral homeostasis (Weiss et al., 2009). The BBB is formed by endothelial cells (ECs) which are themselves sealed by tight junctions (TJs) and adherens junctions (Abbott et al., 2010; Weiss et al., 2009), thus limiting the paracellular diffusion of substances. The BBB also provides a gate-like function for substance delivery to brain cells by limiting trans-cellular diffusion via selective carrier-mediated transport systems (Abbott et al., 2010). An extremely low rate of transendothelial vesicular transport also integrates the transcellular pathway to minimize the uptake of substances from blood to brain parenchyma (Villaseñor et al., 2019). Among the different categories of vesicular transport, caveolae appears to be the major element responsible for transcytosis in cerebral endothelial cells (Villaseñor et al., 2019). At their abluminal side, ECs are surrounded by basal lamina embedding pericytes, and interact with glial cells (astrocytic end-feet and microglial cells) and neurons. The whole is called the neurovascular unit (NVU) (Erdő et al., 2017). The presence of glial cells in the vicinity of the BBB promotes NVU susceptibility to neuro-inflammatory responses. Neuroinflammation, in turn, can contribute to BBB dysfunction and neurodegenerative process (Vodo et al., 2013).

Androgens were also shown to promote cerebral angiogenesis and vasculature formation, and modulate the cerebrovascular function (see for review, Ahmadpour and Grange-Messent, 2021). In particular, our previous data indicate that gonadal testosterone supports the integrity and function of the BBB and prevents gliosis reaction and the up-regulation of inflammatory proteins in adult male mice (Atallah et al., 2017) and rats (Barreto et al., 2007).

In this context, our work aims to document for the first time the impact of oral exposure during adulthood to low doses of DEHP alone or in a phthalate mixture on the integrity of the capillary BBB and surrounding parenchyma in male mice. Analyses were processed using four experimental groups of adult C57BL/6J male mice exposed orally through contaminated diet in order to mimic the major route of exposure as previously described (Adam et al., 2021). The first three groups included males exposed for 6 weeks to the vehicle (control), DEHP at the TDI dose of 50 µg/kg/d, or DEHP at 5 µg/kg/d. The DEHP dose of 5 µg/kg/d is within the environmental exposure range; this dose induced behavioural alterations in male mice following adult or pubertal exposure (Capela and Mhaouty-Kodja, 2021; Dombret et al., 2017). In order to mimic environmental co-exposure to phthalates (Anses, 2015; Martine et al., 2013), the fourth group of males was exposed for 6 weeks to a phthalate mixture containing DEHP at 5 µg/kg/d, DBP at 0.5 µg/kg/d, BBP at 0.5 µg/kg/d, DiBP at 0.5 µg/kg/d and DEP at 0.25 µg/kg/d. Analyses focused on two androgen-sensitive brain areas as mentioned above the hypothalamic mPOA and the hippocampus. In these brain areas, we investigated the effects of exposure on BBB permeability using exogenous tracer and endogenous immunoglobulins G (IgG). Protein levels and distribution of TJ components as well as glial activation and neuroinflammation were also assessed in the four exposed groups.

2. Material and methods

2.1. Ethical statement

The experiments have been reported in compliance with the Animal Research: Reporting in Vivo Experiments (ARRIVE) guidelines. All studies were performed in compliance with the National Institute of Health guidelines for the care and use of Laboratory Animals (NIH Guide) and French and European legal requirements (Decree 2010/63/UE). Experiments were performed accordingly, to minimize animal number and discomfort and were approved by the “Charles Darwin” Ethical committee (project number 01490–01).

2.2. Animals

Males of C57BL/6j strain (Janvier Labs, Le Genest-Saint-Isle, France) bred in our laboratory were housed in a conventional facility after weaning under controlled photoperiod (12:12h light dark cycle-lights on at 1 p.m.), maintained at 22 °C and relative humidity (60% ± 10%), and had free access to water and a standard diet (A03–10; Safe-diets, Augy, France). The mice were housed in nest-enriched polysulfone cages, with polysulfone bottles. Offspring were mixed at the weaning to avoid potential litter effects with no more than one male per litter per cage, and were allowed to grow to 8 weeks of age. For these experiments, 9 cohorts each comprising 9 to 32 animals distributed equally between the 4 treatment groups, were used. Ultimately, 32 animals from two cohorts were used for BBB permeability assays, 96 animals from five cohorts were used for the capillary-enriched fraction procedure and Western blot analysis, and then 68 animals from three cohorts were used for the immunocytochemistry study.

2.3. Phthalate exposure

Exposure to phthalates (Sigma Aldrich, Saint-Quentin Fallavier, France) was performed for 6 weeks as recently described (Adam et al., 2021). The phthalates were first dissolved in absolute ethanol (1% of prepared food) and then in water (40% of prepared food) before incorporated into food as previously described (Adam et al., 2021). Control animals were fed with chow containing the vehicle i.e. ethanol and water (1% and 40% of prepared food, respectively). Eight-week-old males were fed ad libitum with chow consisted of their normal food containing the vehicle (control group), DEHP (CAS 117-81-7) at 50 or 5 µg/kg/d (DEHP-50 and DEHP-5 groups, respectively), or a phthalate mixture (Mix group) containing DEHP at 5 µg/kg/d, DBP (CAS 84-74-2) at 0.5 µg/kg/d, BBP (CAS 85-68-7) at 0.5 µg/kg/d, DiBP (CAS 84-69-5) at 0.5 µg/kg/d and DEP (CAS 84-66-2) at 0.25 µg/kg/d, reconstituted into pellets as precisely described previously (Adam et al., 2021). The composition of the phthalate mixture was based on French and European studies showing an external co-exposure to these molecules and the presence of their metabolites in urinary samples (Anses, 2015; Dewalque et al., 2014; Martine et al., 2013). The ratio of DEHP to the other phthalates was determined on the basis of the estimated daily intake in France and Europe (Dewalque et al., 2014; Martine et al., 2013).

Mice were weighed weekly for the duration of the exposure and phthalate doses were adjusted to their body weights and calculated for a daily food intake of 5 g per animal (Dombret et al., 2017), on the basis of previous studies showing this average daily intake for adult mice of 2–19 months old (Cheema et al., Br J Nutrition, 2019). Body weight was comparable between the four treatment groups on the first and last days of exposure (supplementary Figure S1).

The analyses were performed on 5 cohorts each comprising animals distributed equally between the 4 treatment groups. Briefly, two cohorts were used for BBB permeability assays, two cohorts were used for immunohistochemistry study and then, one was used for the capillary-enriched fraction procedure and Western blot analysis.

2.4. BBB permeability assay

The BBB permeability assay was performed using an exogenous tracer binding to serum albumin, the Evans blue dye (Sigma Aldrich, Saint-Quentin Fallavier, France), and endogenous IgG. The BBB restricts the passage of serum proteins such as albumin and endogenous IgG from the blood flow into the interstitial tissue (Saunders et al., 2015).

2.4.1. Evans Blue dye injection

Three awake mice per treatment group were i.p. injected with a 2% Evans Blue solution (4 ml/kg) diluted in normal saline. Three hours later, the mice were deeply anaesthetized with a lethal dose of pentobarbital (120 mg/kg, i.p.), then transcardially perfused with 0.9% saline solution followed by 4% paraformaldehyde (PFA) solution diluted in 0.1 M phosphate buffer (PB) pH 7.4. Their brains were carefully removed and post-fixed with the same fixation solution overnight at 4 °C. Afterwards, the brains were cryoprotected with a 20% sucrose solution for 24 h at 4 °C before freezing in isopentane (−30 °C) and 8 coronal sections (20 µm thick) included mPOA or hippocampus were cut using a cryostat and mounted on slides. In order to visualize blood vessel walls, all sections were co-stained with anti-laminin rabbit primary antibody (1/100; Table 1) followed by secondary Alexa488-conjugated anti-rabbit IgG (1:1000, Invitrogen, Villebon sur Yvette, France). To avoid the Evans Blue dye spreading and to promote optimal visualization of labeled fine structures, sections were quickly immersed in xylene and mounted with a hydrophobic mounting medium using the method initially developed by Werner et al. (1997), and modified by (Atallah et al., 2017). The Evans blue dye providing a red fluorescence (Steinwall and Klatzo, 1966), fluorescent signals were observed under a confocal microscope (described below).

2.4.2. Endogenous immunoglobulin G detection

Mice (n = 5 per treatment group) were deeply anaesthetized with a lethal dose of pentobarbital (150 mg/kg, i.p.), then were transcardially perfused with 0.9% saline solution followed by 4% PFA solution diluted in 0.1 M PB pH7.4. Their brains were carefully removed and post-fixed with the same fixation solution overnight at 4 °C. Afterwards, the brains were cryoprotected with a 20% sucrose solution for 24 h at 4 °C before freezing in isopentane (−30 °C) and 8 coronal sections (20 µm thick) included mPOA or hippocampus were cut using a cryostat and mounted on slides. The presence of extravasated endogenous mouse IgG from blood to parenchyma was evaluated using Alexa488-conjugated anti-mouse IgG antibody (1:1000, Invitrogen, Villebon sur Yvette, France) combined with immuno-labelling of laminin as described above to detect microvessels. Then fluorescent staining was observed under a confocal microscope (described below).

2.5. Fluorescent immunohistochemistry

The animals (n = 5 per treatment group and per immuno-labelling) were deeply anaesthetized with a lethal dose of pentobarbital (120 mg/kg, i.p.) for the following procedures.

2.5.1. Tissue preparation

The experiments were processed as previously described (Atallah et al., 2017). Briefly, for TJ proteins (claudin-5, occludin and ZO-1) and the main component of caveolae plasma membranes, Cav-1 protein, the brains were freshly removed and immediately frozen in isopentane (−30 °C). Six to eight serial frozen sections (20-µm thickness) included mPOA and hippocampus were cut and collected on slides, and were then fixed by immersion for 2 min at −20 °C in methanol/acetone (vol/vol) with this followed by the labelling procedure. For the other proteins (Iba-1, GFAP, S100β, NDRG-2, Cox-2 and iNOS), male mice were deeply anaesthetized using i.p. injection of pentobarbital (120 mg/kg) diluted in with 0.9% saline solution and then transcardially perfused with 0.9% saline solution followed by 4% paraformaldehyde solution diluted in 0.1 M PB pH7.4. After post-fixation and cryoprotection steps as described above, the brains were frozen in isopentane (−30 °C) and 6 to 8 serial frozen sections (20 µm thickness) included mPOA or hippocampus were cut using a cryostat and collected on slides.

2.5.2. Immuno-labelling procedure

Non-specific sites were blocked by incubating slide-mounted sections in PBS 1X, 1% bovine serum albumin (BSA) and 0.2% Triton X-100 for 1 h at room temperature. Then sections were incubated with one or more primary antibodies (Table 1) overnight at 4 °C diluted in the same phosphate buffer saline (PBS)/bovine serum albumin (BSA)/Triton X-100 solution. Immune complexes were revealed using secondary Alexa-conjugated anti-mouse, anti-goat or anti-rabbit IgG (1:1000; Invitrogen, Villebon sur Yvette, France). Fluorescence was observed with a confocal microscope.

2.6. Confocal microscopy

Simple and multiple fluorescent labelling was visualized with a SP5 upright Leica confocal laser scanning microscope (Leica Microsystems) equipped with the Acousto-Optical Beam Splitter (AOBS) and using 63× oil immersion objective. Alexa 488 was excited at 488 nm and observed from 495 to 580 nm; Alexa 555 was excited at 555 nm and observed from 599 to 680 nm. The gain and offset for each photomultiplier were adjusted to optimize detection events. Images (1024x1024 pixels, 16 bits) were acquired sequentially between stacks to eliminate cross-over fluorescence. The frequency was set up at 400 Hz and the pinhole was

Table 1
List of primary antibodies.

Antibody	Host	Manufacturer	Catalog no	Application	Working dilution
NDRG2	Rabbit	Cell Signaling Technology	56675	IHC	1/400
S100β	Mouse	Sigma-Aldrich	s2532	IHC	1/1000
COX-2	Goat	Santa Cruz	Sc-1747	IHC - WB	1/200
Iba-1	Rabbit	WAKO	016-20001	WB	1/500
Iba-1	Rabbit	Biocare Medical	CP-290	IHC	1/300
ZO-1	Rabbit	Invitrogen	61-7300	IHC - WB	1/125–1/500
Claudin-5	Rabbit	Invitrogen	34-1600	IHC - WB	1/500
Cav-1	Mouse	Biosciences	610,407	IHC	1/200
Cav-1	Mouse	Santa Cruz	sc-53564	WB	1/200
iNOS	Mouse	Santa Cruz	sc-7271	WB	1/200
iNOS	Mouse	Sigma-Aldrich	n9657	IHC	1/1000
GFAP	Mouse	Sigma-Aldrich	G3893	WB	1/500
GFAP	Rabbit	DAKO	Z0334	IHC	1/500
Laminin	Rabbit	Sigma-Aldrich	L9393	IHC - WB	1/200–1/100
Occludin	Rabbit	Invitrogen	40-4700	IHC - WB	1/500–1/250
GAPDH	Mouse	Santa Cruz	sc-32233	WB	1/10,000

IHC: Immunohistochemistry; WB: Western blotting.

set at 1 Airy. Each optical section (1 μm) was frame-averaged four times to enhance the signal/noise ratio. Overlays, projection of the z-stack files and quantification were performed using the Fiji software (NIH, USA). The presented pictures were the projection of 10–20 successive optical sections into one image, unless otherwise stated in the figure legend. Quantification of the fluorescent density was performed on three sections sampled at the level of the mPOA (plate 30 of the Mouse Brain Atlas of Paxinos and Franklin 2001) and on three sections sampled at the level of the hippocampus (plate 48). The surfaces on which the fluorescence density quantifications were performed are given in the figure legends.

2.7. Western blot protein analysis using cerebral capillary-enriched fractions

2.7.1. Cerebral capillary-enriched fraction procedure

Brains from 12 mice per treatment group were freshly removed and placed quickly on ice. For each hypothalamic capillary-enriched fraction, two hypothalami were pooled ($n = 6$ per treatment group), whereas one hippocampus was used for each hippocampal capillary-enriched fraction ($n = 6$ per treatment group). Hypothalamic and hippocampal microvessels were harvested according to a previously described method (Atallah et al., 2017; Sandoval and Witt, 2011), in order to obtain microvessel-enriched fractions for each cerebral area. Briefly, samples were homogenized in a pH 7.4 buffer containing 1% BSA, 2.7 mM KCl, 137 mM NaCl, 1.5 mM KH_2PO_4 , 8 mM Na_2HPO_4 , 1 mM CaCl_2 , 0.5 mM MgCl_2 , 6H₂O, mM D-glucose, 1 mM sodium pyruvate, 1 M 4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES). Homogenates were centrifuged in an equal volume of 30% Ficoll for 15 min at 4800g at 4 °C, supernatants were aspirated and pellets suspended in isolation buffer without BSA and passed through a 70- μm nylon filter. Filtrates were centrifuged for 10 min at 4 °C at 3000g.

2.7.2. Protein extraction

Protein extraction from pellets of microvascular capillary fractions was performed with a RIPA buffer containing 50 mM Tris—base (pH 7.2), 10 mM EDTA, 10 mM EGTA, 150 mM NaCl, 0.1% sodium dodecyl sulfate, 0.5% deoxycholate acid, 1% Triton X-100, and 1% protease inhibitor cocktail (Sigma Aldrich) and sonicated 10 times for 30 s. Homogenate samples were centrifuged at 13,000 rpm for 13 min at 4 °C and supernatants containing proteins were collected. The total protein concentration of each sample was determined using the Bradford Assay Kit (Thermo Scientific, Courtaboeuf-Villebon sur Yvette, France) according to the manufacturer's protocol. Protein extracts were stored at –20 °C until further processing.

2.7.3. Electrophoresis and immunoblotting

Protein samples were denatured in Laemmli Buffer and heated at 95 °C for 5 min. Electrophoretic migration of 10–20 μg of proteins was carried out on NuPAGE 4–12% Bis–Tris Gel (Invitrogen, Villebon sur Yvette, France) or Mini-PROTEAN® TGX™ 7.5% polyacrylamide gels (BIO-RAD, Marnes La Coquette, France). The resolved proteins were then electrotransferred onto pre-treated polyvinylidene difluoride (PVDF) membranes (Millipore, Molsheim, France).

Membranes with transferred proteins were blocked for 1 h, at RT with a solution of 5% non-fat milk diluted in PBS 1X with 0.2% Tween, and then incubated with primary antibodies (Table 1) diluted in the same blocking solution overnight at 4 °C. Primary antibody binding to blots was detected by incubation with respectively either secondary HRP-conjugated (1:5000; Jackson, Cambridgeshire, United Kingdom) or biotin-conjugated (1:2000; Vector, Burlingame, United States) anti-rabbit, anti-mouse or anti goat for 2 h, at RT, and then immune complexes were revealed by the SuperSignal™ West Pico or Femto Chemiluminescent Substrate kit (Thermo Scientific, Courtaboeuf-Villebon sur Yvette, France).

The signals were quantified by using Fiji software (NIH, USA) and

normalized to the value obtained for the corresponding housekeeper glyceraldehyde- 3-phosphate dehydrogenase (GAPDH) protein band.

2.8. Statistical analysis

The four sample sizes corresponding to the vehicle-treated, DEHP-5 or DEHP-50 and phthalate mixture were equal. Normal distribution of the four groups was checked using the Shapiro-Wilk normality test then one-way ANOVA was used to analyze the main effects of exposure and Tukey tests were used for posthoc analyses to determine group differences. Differences were considered statistically significant if $p \leq 0.05$. In the text, means \pm S.E.M are expressed as a percentage of the vehicle group. Two-way ANOVA was used to analyze the effects of treatment on the body weight of exposed mice. Data are expressed as body weight means (g) \pm S.E.M and differences were considered statistically significant if $p \leq 0.05$.

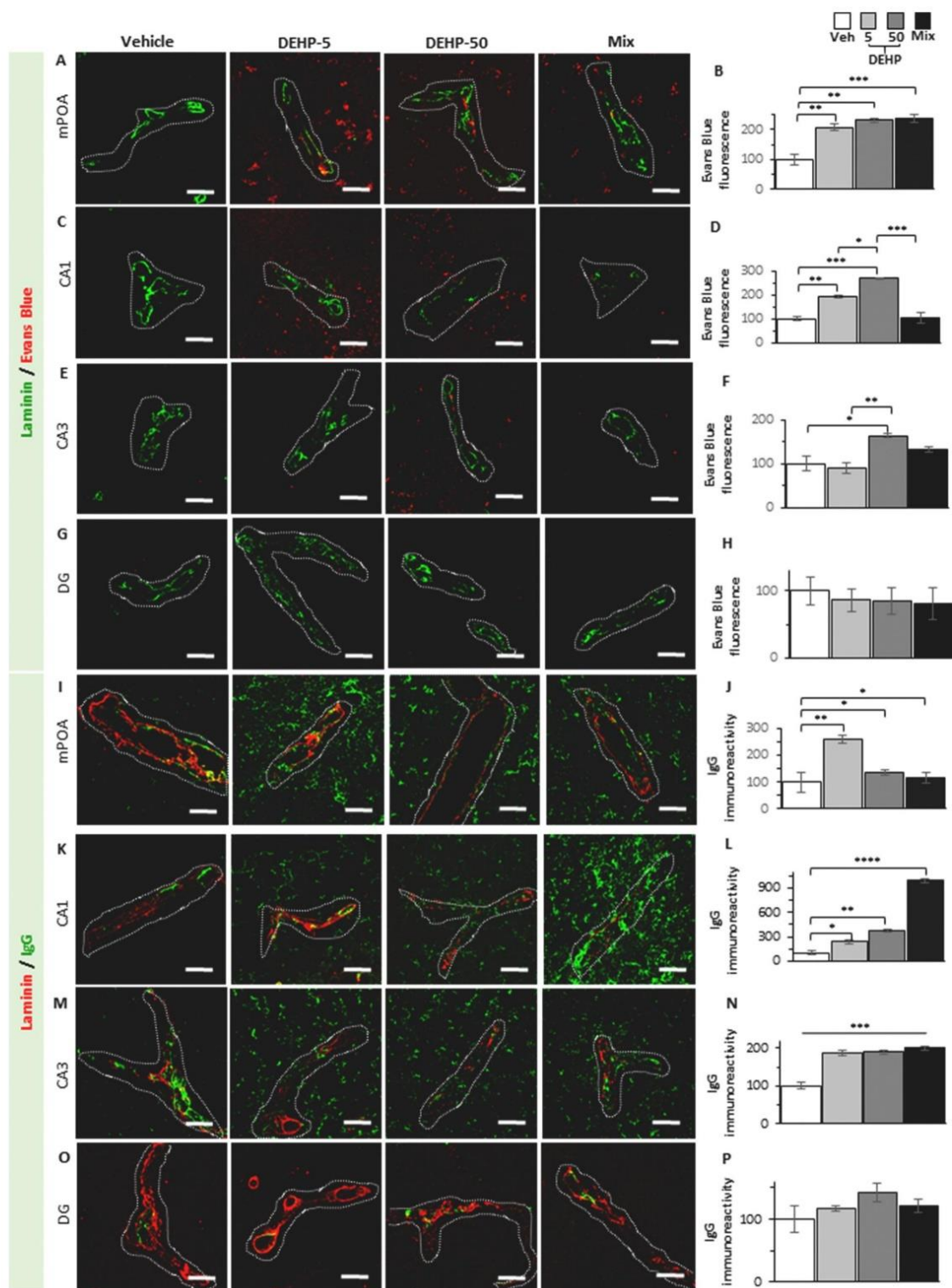
3. Results

3.1. BBB permeability

BBB permeability in the hypothalamic mPOA and hippocampal CA1, CA3 and DG sub-regions was assessed in mice exposed to the vehicle or DEHP alone or in a phthalate mixture by using exogenous Evans Blue dye (Fig. 1; A–H), which binds to albumin protein, and endogenous circulating IgG molecules (Fig. 1; I–P). Detection of these tracers in the parenchyma, called extravasation, indicates a BBB leakage. Therefore, to visualize blood capillary walls, laminin, the main basal lamina protein, was immunodetected.

In the hypothalamic mPOA, fluorescent signal analyses showed treatment had had a significant effect ($p \leq 0.01$) on the extravasation of the Evans Blue dye outside perfused brain capillaries, with an increased extravasation in DEHP-5-, DEHP-50- and Mix-treated mice ($+110\%$ $p \leq 0.01$, $+130\%$ $p \leq 0.01$ and $+135\%$ $p \leq 0.001$, respectively, above vehicle-treated mice) determined by posthoc analyses (Fig. 1A and B). In the hippocampus, the treatment was found to have had an effect on the extravasation of the Evans Blue dye in the CA1 and CA3 regions ($p \leq 0.0001$, $p \leq 0.01$, respectively). A significant increase above vehicle-treated mice was evidenced by posthoc analyses for DEHP-5- and DEHP-50-treated mice in the CA1 parenchyma ($+95\%$ $p \leq 0.01$ and $+170\%$ $p \leq 0.001$ respectively, $p \leq 0.05$), but only for DEHP-50-treated mice in the CA3 parenchyma ($+70\%$, $p \leq 0.05$) (Fig. 1C–F). In contrast, there was no treatment effect in the DG capillaries, which greatly restricted Evans blue dye extravasation to the brain parenchyma (Fig. 1G and H).

Immunolabelling of endogenous circulating IgG showed that endogenous IgG were observed and were restricted to capillary wall or lumen in vehicle-treated mice (Fig. 1K and M). In contrast, the effect of treatment on IgG immunoreactivity in the mPOA ($p \leq 0.01$), with higher levels in DEHP-5, DEHP-50- and mixture-treated mice ($+160\%$, $p \leq 0.01$; $+35\%$ and $+15\%$, $p \leq 0.05$; respectively) in the parenchyma of phthalate-treated mice compared to the vehicle group (Fig. 1I and J) was shown by posthoc analyses. Similarly, a treatment effect ($p \leq 0.0001$) was found in the CA1 area with significantly higher levels of endogenous IgG in the DEHP-5 ($+140\%$, $p < 0.05$), DEHP-50 ($+270\%$, $p < 0.01$) and Mix-groups ($+900\%$, $p < 0.0001$) versus the vehicle group (Fig. 1K and L) was shown by posthoc analyses. There was also a treatment effect in the CA3 area ($p \leq 0.0001$) as illustrated in Fig. 1M and N; posthoc analyses showed increased levels for mice exposed to DEHP alone or in a mixture ($+86\%$, $+89\%$ and $+100\%$, $p \leq 0.0001$, for DEHP-5, DEHP-50 and mixture-group vs. vehicle, respectively). In accordance with the result obtained for Evans blue dye, no extravasation of circulating IgG from blood compartment to hippocampal DG parenchyma was detected (Fig. 1O and P).



(caption on next page)

Fig. 1. Exposure to DEHP alone or in a phthalate mixture increased BBB permeability to Evans blue dye and endogenous IgG. The permeability of BBB was analyzed using Evans blue dye ($n = 3$ per group, A to H) and immunolabeled endogenous IgG ($n = 5$ per group, I to P) in the hypothalamic medial preoptic area (mPOA) and hippocampal CA1, CA3 and DG. Images were selected from brain sections containing the sub-regions of interest in adult male mice orally exposed to vehicle (Veh; first column), DEHP at 5 $\mu\text{g/kg/d}$ (DEHP-5; second column), DEHP at 50 $\mu\text{g/kg/d}$ (DEHP-50; third column) and a phthalate mixture (Mix; fourth column). (A–H) Representative images and corresponding quantitative analysis of the six to eight serial sections for each brain examined of the mPOA (A, B), CA1 (C, D), CA3 (E, F) and DG (G, H) for Evans blue dye tracer (red), capillaries were labeled with anti-laminin (green). (I–P) Representative images and corresponding quantitative analysis of the six to eight serial sections for each brain examined of the mPOA (I, J), CA1 (K, L), CA3 (M, N) and DG (O, P) of endogenous circulating IgG (green), capillaries were labeled with anti-laminin (red). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$ compared to vehicle-treated mice compared to the vehicle group. All data are expressed as mean percentages \pm S.E.M of vehicle (100%). The pictures presented are the projection of 3 successive optical sections (1 μm) into one image. Scale bar: 10 μm . The dotted lines delimit the outer contour of the capillaries. The quantifications of fluorescence density were measured over the entire surface of the images. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.2. Protein levels and distribution of TJ components

BBB leakage induced by DEHP alone or in a mixture could be due to an alteration of capillary inter-endothelial TJs leading to paracellular transport of components. TJs comprise a complex of transmembrane proteins claudin-5 and occludin, which are associated with peripheral scaffolding proteins. Therefore, we investigated the distribution and amounts of these proteins using immunofluorescence and Western blot analysis on microvessel-enriched fractions. No significant effect of treatment was found on the protein amounts of claudin-5 and occludin in the capillaries of hypothalamus (Fig. 2A and B) and hippocampus (Fig. 2D and E). In addition, confocal microscopy analysis of the capillaries of hypothalamic mPOA and hippocampal CA1, CA3 and DG sub-regions, showed no significant treatment effect on the distribution of immuno-labelling of claudin-5 (supplementary figure S2) and occludin (supplementary Figure S3).

Those endothelial transmembrane components of TJs interact with the actin cytoskeleton via cytoplasmic TJs accessory proteins with the ZO-1 protein having predominance. Data from Western blot performed on microvessel-enriched fractions obtained from the hypothalamus

(Fig. 2C), highlighted a treatment effect on ZO-1 protein amount ($p \leq 0.0001$), with less ZO-1 for both DEHP-5- and DEHP-50-treated mice (-75% , $p \leq 0.0001$) and also Mix-treated mice (-65% , $p \leq 0.0001$) versus the vehicle group following posthoc analyses. A treatment effect was also found on the amount of ZO-1 in hippocampal capillaries (Fig. 2F). Posthoc analyses showed that the amount of ZO-1 was significantly increased for DEHP-50- treated mice ($+50\%$, $p \leq 0.05$), whereas no significant difference was detected for DEHP-5- and mixture-treated groups compared to vehicle (Fig. 2F). As for claudin-5 and occludin, confocal microscopy analysis of the capillaries of hypothalamic mPOA and hippocampal CA1, CA3 and DG sub-regions, showed no treatment effect on the immuno-labelling of ZO-1 protein (supplementary Figure S4).

3.3. Cav-1 protein level in capillaries walls

Caveolae are non-clathrin-coated vesicles with plasma membranes enriched in caveolin (Cav) proteins, involved in the receptor-independent transcellular transport of molecules from blood to parenchyma. We analyzed the level of caveolin-1 isoform protein (Cav-1), the

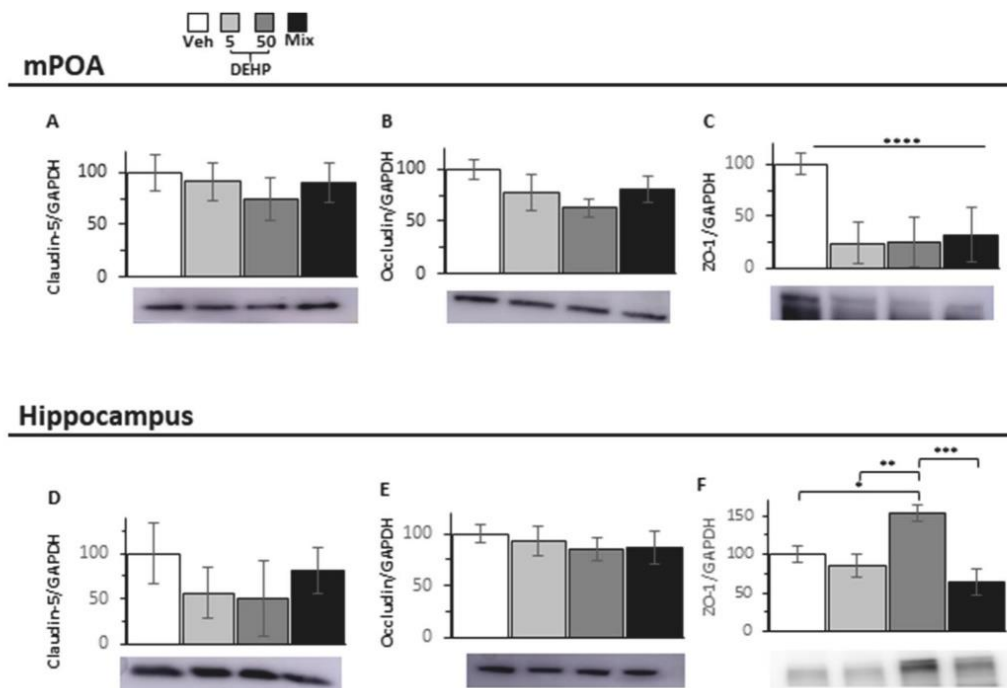


Fig. 2. Exposure to DEHP alone or in a phthalate mixture affected endothelial tight junction protein ZO-1 levels but not claudin-5 and occludin protein levels. Western blot analysis ($n = 6$ per treatment group) of tight junction proteins performed on microvessel-enriched fractions from hypothalamus (A–C) and hippocampus (D–F) of mice exposed to the Vehicle (Veh), DEHP at 5 $\mu\text{g/kg/d}$ (DEHP-5), DEHP at 50 $\mu\text{g/kg/d}$ (DEHP-50) or the phthalate mixture (Mix). All data are expressed as mean percentages \pm S.E.M of vehicle (100%). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$ compared to the vehicle group. Data were normalized to GAPDH level.

main component of the caveolae plasma membranes. Fluorescence analysis revealed an effect of treatment ($p \leq 0.01$) on Cav-1 immunoreactivity in capillary walls and posthoc analyses showed a significant high decrease for DEHP-50- and Mix-treated mice in the mPOA (DEHP-50 group: 50%; $p < 0.05$ and mixture-group: 75%; $p < 0.01$ vs. vehicle;

Fig. 3A and B) and in the CA-1 (DEHP-50 group: 60%; $p < 0.05$ and mixture-group: 70%; $p < 0.01$ vs. vehicle; Fig. 3D and E) and CA-3 areas ($\sim 70\%$; $p < 0.01$ vs. vehicle in DEHP-50 and mixture-group; Fig. 3F and G). Again, no significant treatment effect was found in hippocampal DG (Fig. 3H and I). These results were confirmed by Western blot analysis

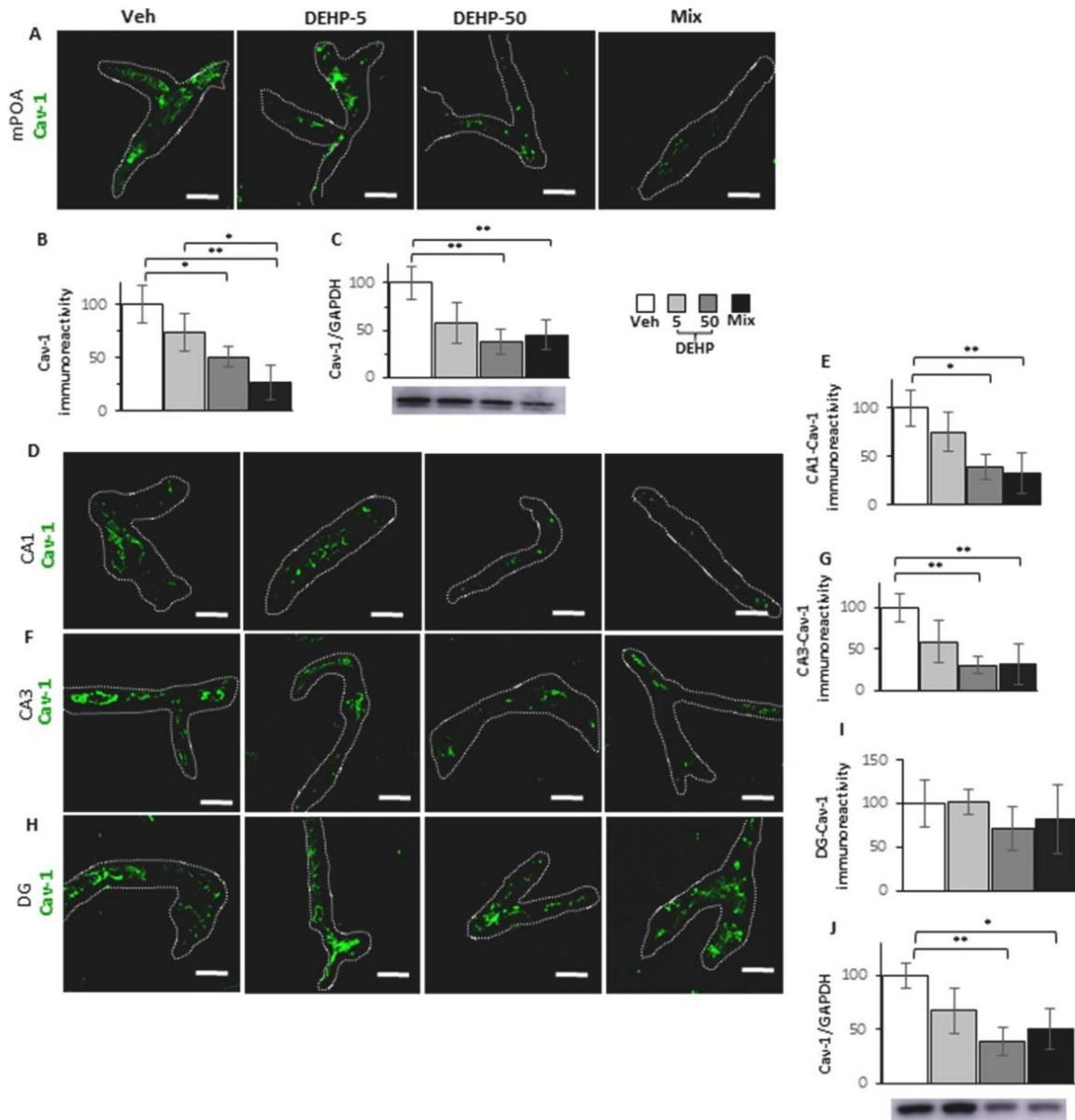


Fig. 3. Exposure to DEHP alone or in a phthalate mixture decreased protein levels of the caveolae-associated membrane protein Cav-1. Images were selected from brain sections containing the sub-regions of interest in adult male mice orally exposed to vehicle (Veh; first column), DEHP at 5 $\mu\text{g}/\text{kg}/\text{d}$ (DEHP-5; second column), DEHP at 50 $\mu\text{g}/\text{kg}/\text{d}$ (DEHP-50; third column) and a phthalates mixture (Mix; fourth column). (A, B) Representative images of immunodetection of Cav-1 in the mPOA (A) and the corresponding quantitative analysis of the Cav-1 immunoreactivity density (B) of the six to eight serial sections for each brain examined ($n = 5$ per treatment group). Values represent mean percentages \pm S.E.M of vehicle (100%). * $p < 0.05$; ** $p < 0.01$ compared to the vehicle group. (C) Western blot analysis of Cav-1 performed on microvessel-enriched fractions. * $p < 0.05$; ** $p < 0.01$ compared to the vehicle group ($n = 6$ per treatment group). Data were normalized to GAPDH levels. (D–I) Representative images of immunodetection of Cav-1 in the hippocampus (D: CA1; F: CA3; H: DG) and the corresponding quantitative analysis of the Cav-1 immunoreactivity density (E: CA1; G: CA3; I: DG) of the six to eight serial sections for each brain examined ($n = 5$ per group). * $p < 0.05$; ** $p < 0.01$ compared to the vehicle group. (J) Western blot analysis of Cav-1 performed on microvessel-enriched fractions from hippocampus. * $p < 0.05$; ** $p < 0.01$ compared to the vehicle group ($n = 6$ per treatment group). Data were normalized to GAPDH level. Scale bar: 10 μm . The dotted lines delimit the outer contour of the capillaries. The quantifications of fluorescence density were measured over the entire surface of the images. All values represent mean percentages \pm S.E.M of vehicle (100%).

performed on microvessel-enriched fractions showing a treatment effect ($p \leq 0.001$), with a decrease by -63% and -56% ($p < 0.01$ in DEHP-50 and mixture-group, respectively) in hypothalamic vessels (Fig. 3C) and -60% ($p < 0.01$) and -50% ($p < 0.05$) in hippocampal vessels of DEHP-50 and mixture-group respectively in comparison to the vehicle group (Fig. 3J) determined by posthoc analyses.

3.4. Glial activation and neuroinflammation

The neuroinflammation process occurs following several brain insults and is often associated with a dysfunction of the neurovascular unit. Neuroinflammatory responses are underlined on the one hand by a glial activation involving an increase of glial specific protein levels, NDRG2, S100 β and GFAP for astrocyte, and Iba-1 for microglia, and on the other hand by an increase of inflammatory molecules such as iNOS and COX-2. The distribution of these proteins was assessed using immunofluorescence, and protein levels in glial cells closely associated with capillaries were assessed using Western blot analysis performed on microvessel-enriched fractions from the whole hypothalamus and hippocampus.

In the mPOA, analyses of immunofluorescence results showed an effect of treatment on NDRG2 and S100 β labellings ($p \leq 0.0001$; $p \leq 0.05$, respectively). A significant increase of NDRG2 labelling in astrocytes for mixture-treated mice ($+125\%$, $p < 0.0001$ vs. vehicle, Fig. 4A and B) and a significant increase of S100 β labelling in astrocytes for DEHP-50- and mixture-treated mice ($+92\%$ and $+82\%$ vs. vehicle, respectively, $p < 0.05$, Fig. 4A and C) were determined by posthoc analyses. All NDRG2-positive astrocytes are immunoreactive for S100 β but few S100 β -positive astrocytes express NDRG2. This astrocytic activation was confirmed by a significant enhancement of GFAP immunofluorescence density in the parenchyma and in the vicinity of capillaries for DEHP-50-treated mice ($+100\%$; $p < 0.05$ vs. vehicle, Fig. 4D and E). By contrast, Western blot analysis performed on microvessel-enriched fractions showed an effect of treatment ($p \leq 0.0001$), with a significant decrease of GFAP amount in the astrocytic end-feet which remained in contact with the isolated vessels for DEHP-5-, DEHP-50- and mixture-treated mice compared to vehicle (-40% , $p < 0.01$, -55% , $p < 0.0001$ and -37% , $p < 0.01$, respectively vs. vehicle, Fig. 4G), determined by posthoc analyses.

Immunofluorescent analysis showed an effect of treatment ($p \leq 0.01$), with a stronger signal for iNOS colocalized with GFAP surrounding capillaries not only for DEHP-5-, DEHP-50-treated mice ($+280\%$; $p < 0.01$ vs. vehicle) but also for mixture-treated mice compared to vehicle ($+360\%$; $p < 0.001$ vs. vehicle) (Fig. 4D and F). Conversely, no significant effect was highlighted for iNOS protein level using Western blot analysis performed on hypothalamic microvessel-enriched fractions (Fig. 4H). Immunohistochemical labelling for the specific marker of microglial cells, Iba-1, was also changed by the treatment ($p \leq 0.01$) and posthoc analyses showed a significant increase in the mPOA parenchyma for DEHP-50-treated mice ($+125\%$; $p < 0.01$ vs. vehicle, Fig. 6A and B), whereas hypothalamus capillary-enriched fraction showed no difference in the amount of Iba-1 protein among the experimental groups (Fig. 6C). COX-2 protein was immuno-detected in Iba-1-immunopositive microglial cells in the mPOA (Fig. 6A) especially for DEHP-50-treated group compared to vehicle ($+130\%$; $p < 0.01$, Fig. 6D). However, no significant difference in the COX-2 protein amount was measured in the capillaries of hypothalamus of phthalate-treated mice compared to those of vehicle-treated mice (Fig. 6E).

In the hippocampus, analysis of the environment of the capillaries of CA1, CA3 and DG sub-regions showed no significant treatment effect for immuno-labelling intensity of NDRG2 and S100 β for all phthalate-treated groups compared to the vehicle (Fig. 5A–F). Observations using confocal microscopy confirmed these results (supplementary figure S5). Quantification of GFAP immunoreactivity displayed no significant treatment effect between control and phthalate-treated mice not only in the CA1 (Fig. 5G and H) but also in the CA3 (Fig. 5J and K) and in

the DG region (Fig. 5M and N). Western blot analysis performed on hippocampal microvessel-enriched fractions did not show any modification of GFAP protein content in the hippocampus, regardless of the experimental group (Fig. 5P). Immunofluorescent analysis showed that iNOS labelling was not co-localized with GFAP labelling in the CA1 region (Fig. 5G), but was significantly impacted by the treatment ($p \leq 0.01$) and enhanced for DEHP-5-, DEHP-50 and mixture-treated mice compared to the vehicle ($+180\%$; $p < 0.01$, $+125\%$; $p < 0.05$, and $+115\%$; $p < 0.05$, respectively vs. vehicle, Fig. 5I) determined by posthoc analyses. In the hippocampal CA3 region, the level of iNOS immunoreactivity, not detected in astrocyte (Fig. 5J), was equivalent between animals treated with the vehicle and DEHP alone or in a mixture (Fig. 5L). In the hippocampal DG, iNOS immunoreactivity was affected by the treatment ($p \leq 0.001$) and significantly increased only for DEHP-5-treated mice ($+270\%$; $p < 0.001$, vs. vehicle, Fig. 5O). Finally, no effect on iNOS protein amount was measured (Fig. 5Q) in the hippocampal microvessels.

Iba-1 immunoreactivity was affected by treatment ($p \leq 0.05$) and increased in the CA1 ($+115\%$, $+125\%$ and $+118\%$; $p < 0.05$ vs. vehicle in DEHP-5, DEHP-50 and mixture-group respectively; Fig. 6F and G), and in CA3 areas (DEHP-5 group: $+200\%$; $p < 0.0001$; DEHP-50 group: $+90\%$; $p < 0.05$; mixture-group: $+80\%$; $p < 0.05$ vs. vehicle; Fig. 6I and J). No treatment effect was measured in the DG (Figure L and M). In the hippocampal capillaries-enriched fractions, no significant treatment effect was observed on the protein amounts of Iba-1 (Fig. 6O). Confocal microscopy and Western blot analysis showed no significant effect on COX-2 immunolabelling (Fig. 6H, K and N) and the amount of protein in microvessel-enriched fractions of the hippocampus (Fig. 6P).

4. Discussion

This study aimed to characterize the impact of a subchronic oral exposure to environmental doses of DEHP alone or in a phthalate mixture on the neurovascular unit in the hypothalamus and hippocampus of adult male mice. To our knowledge of the available literature, this study is the first to assess the effects of adult exposure to low doses of phthalates on the blood-brain barrier.

The data obtained show that exposure to DEHP alone or in a phthalate mixture significantly increased the BBB permeability in the hypothalamic mPOA and the hippocampal CA1 and CA3 regions as evidenced by the use of two different techniques assessing extravasation of exogenous Evans blue tracer and circulating endogenous IgG. It is generally accepted that increased permeability of the BBB reflects the failure of its functional integrity (Erdő et al., 2017), which can be manifested by an alteration at the molecular and/or morphological level of the constituents of these TJs (Obermeier et al., 2013). Our results show that exposure to DEHP alone or in a phthalate mixture did not affect the expression of the major TJs transmembrane proteins, claudin-5 and occludin, indicating that the increased BBB permissiveness was not due to changes in these protein amounts in the two brain structures we assessed. Our results show that the increased BBB permeability induced by exposure to DEHP alone at 5 or 50 $\mu\text{g/kg/d}$ or DEHP 5 $\mu\text{g/kg/d}$ in a phthalate mixture was associated with a dramatically decreased amount of the accessory junctional protein ZO-1 in hypothalamic microvessel-enriched fractions. An opposite effect was observed in hippocampal microvessel-enriched fractions in the group exposed to DEHP at 50 $\mu\text{g/kg/d}$. The underlying cause of these changes could, at least in part, be the observed BBB integrity failure induced by exposure to DEHP alone or in a mixture, since interaction between claudin, occludin and ZO-1 is pivotal for facilitating tight junction assembly, and regulating the effectiveness of TJs complex function (Abbott et al., 2010). However, it is important to note that no difference was detected in ZO-1 immunoreactivity when assessed by immunohistochemistry in the mPOA, or CA1 and CA3 regions. A possible explanation could be that in the brain sections submitted to immunohistochemical analyses, the quantifications were carried out

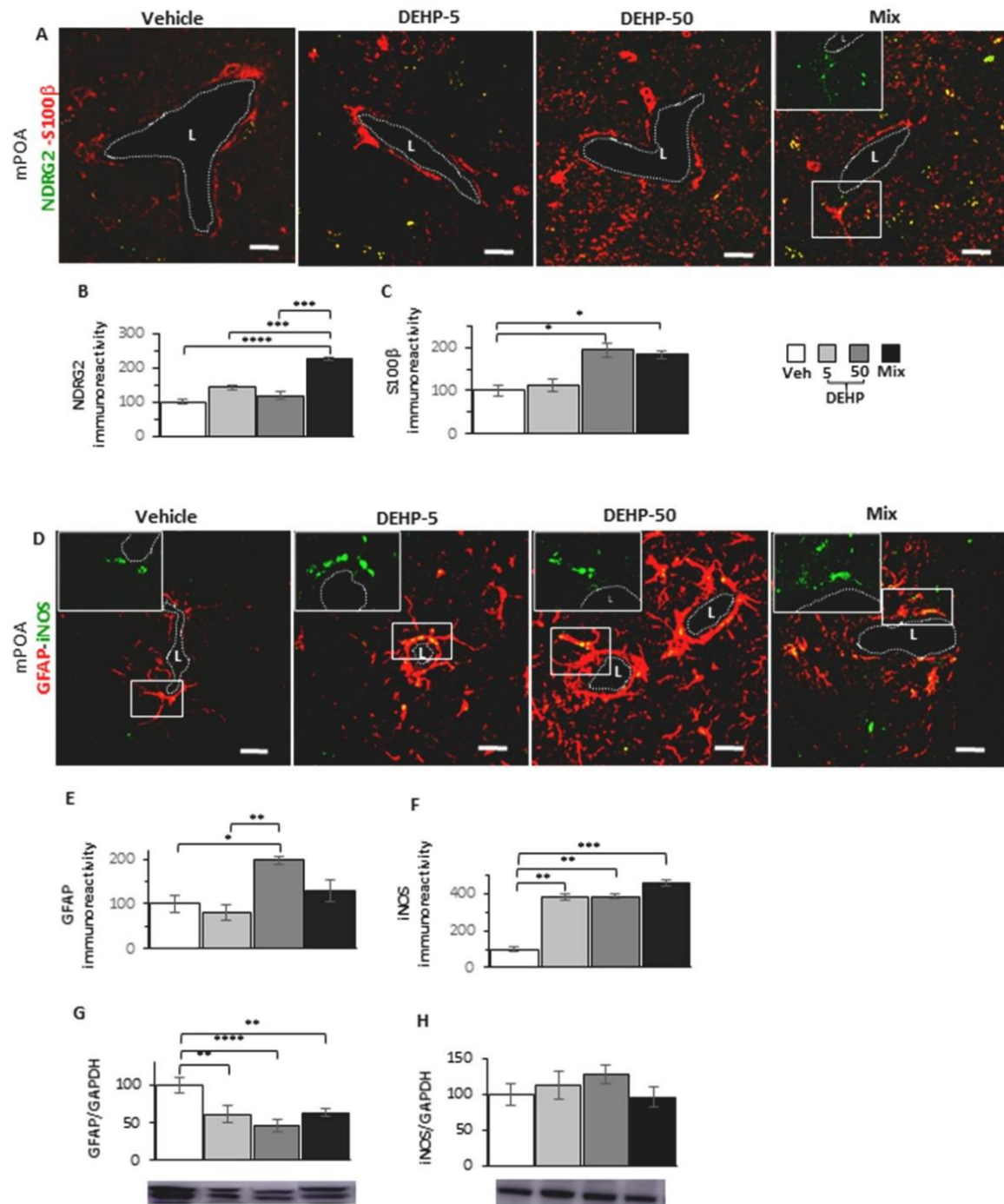
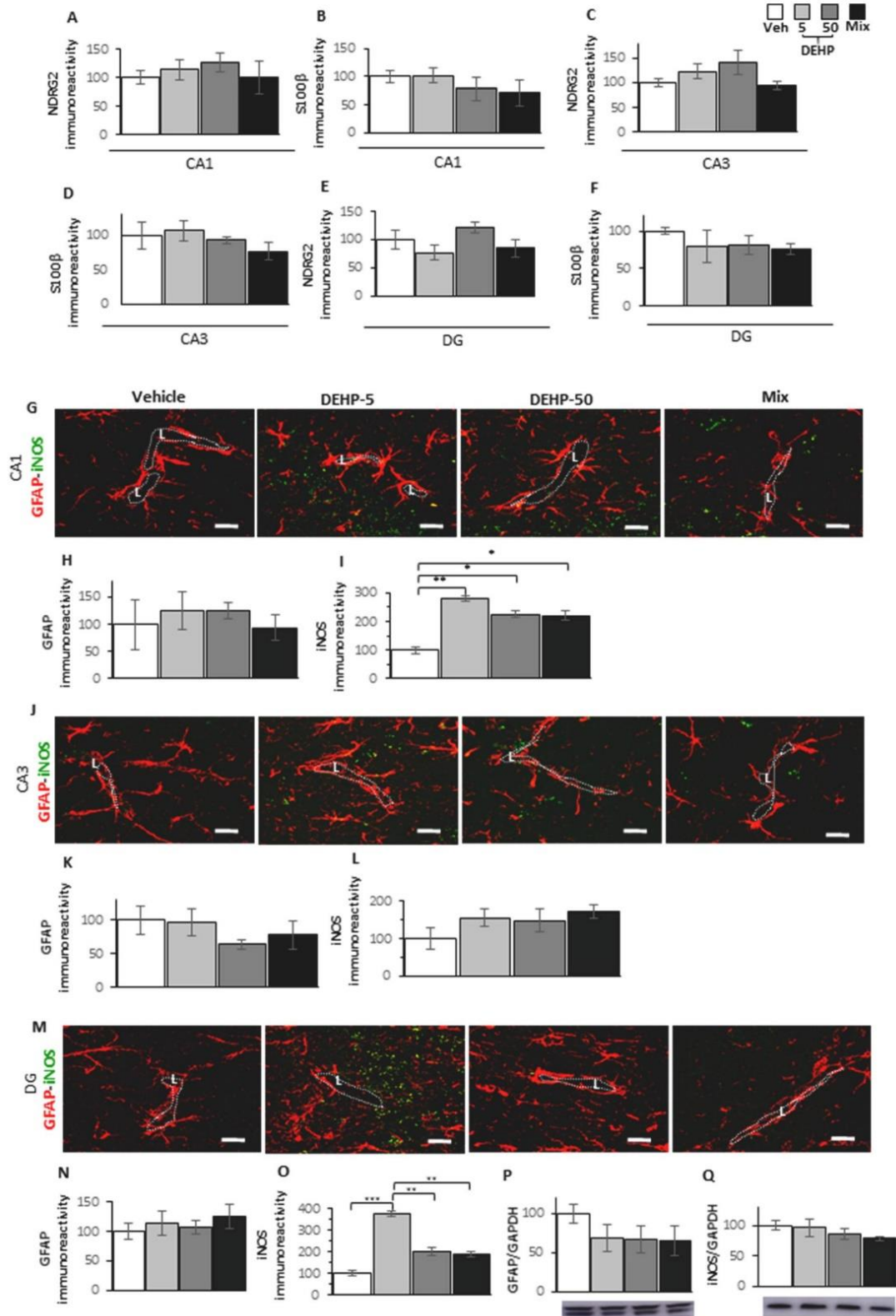


Fig. 4. Exposure to DEHP alone or in a phthalate mixture induced astrocyte activation and iNOS expression in the medial preoptic area (mPOA). (A–C) Representative images (A) and corresponding quantitative analysis (B, C) of the six to eight serial sections for each brain examined of the co-immunolabeling of NDRG2 (green, insert) and S100 β (red) in the mPOA of mice exposed to the vehicle (Veh), DEHP at 5 μ g/kg/d (DEHP-5), DEHP at 50 μ g/kg/d, or phthalate mixture (Mix) (n = 5 per treatment group). (D–F) Representative images (D) and corresponding quantitative analysis (E, F) of the six to eight serial sections for each brain examined of the co-immunolabeling of iNOS (green, inserts) and GFAP (red) in the mPOA (n = 5 per group). * p < 0.05; *** p < 0.001; **** p < 0.0001 compared to the vehicle group. (G, H) Western blot analysis (n = 6 per treatment group) of GFAP (G) and iNOS (H) performed on microvessel-enriched fractions from the hypothalamus. * p < 0.05; ** p < 0.01; *** p < 0.001; **** p < 0.0001 compared to the vehicle group (n = 6 per group). Data were normalized to GAPDH level. Scale bar: 10 μ m. L: Lumen delimited by the dotted lines. The quantifications of fluorescence density were measured over the entire surface of the images. All values represent mean percentages \pm S.E.M of vehicle (100%). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)



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Fig. 5. Exposure to DEHP alone or in a phthalate mixture had no effect on astrocyte activation in the hippocampus but induced iNOS expression in the CA1 and DG. (A–F) Quantitative analysis of the immunoreactivity of NDRG2 and S100 β in the hippocampus (A, B: CA1; C, D: CA3; E, F: DG) of the six to eight serial sections for brain collected from mice exposed to the vehicle (Veh), DEHP at 5 $\mu\text{g/kg/d}$ (DEHP-5), DEHP at 50 $\mu\text{g/kg/d}$, or phthalate mixture (Mix) ($n = 5$ per treatment group). No significant difference was measured compared to vehicle-treated controls. (G–O) Representative images of co-immunodetection of GFAP (red) and iNOS (green) in the hippocampus (G: CA1; J: CA3; M: DG) and their corresponding quantitative analysis of the immunoreactivity density (H, I: CA1; K, L: CA3; N, O: DG) of the six to eight serial sections for each brain examined ($n = 5$ per group). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ compared to vehicle-treated controls. (P, Q) Western blot analysis ($n = 6$ per group) of GFAP (P) and iNOS (Q) performed on microvessel-enriched fractions from hippocampus. Data were normalized to GAPDH level. No significant difference was measured compared to vehicle-treated controls. Scale bar: 10 μm . L: Lumen delimited by the dotted lines. The quantifications of fluorescence density were measured over the entire surface of the images. All values represent mean percentages \pm S.E.M of vehicle (100%). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

from 2-dimensional images and therefore the absence of the third dimension may impede the complete evaluation of the components of TJ proteins.

In addition to this possible impairment in the organization of endothelial TJs, our results also highlight potential modifications in caveolae-mediated transcellular transport. Indeed, adult exposure to DEHP at 50 $\mu\text{g/kg/d}$ or to DEHP 5 $\mu\text{g/kg/d}$ in the phthalate mixture led to a significant decrease in Cav-1 immunoreactivity in the mPOA and CA1 and CA3 regions, and in the amount of protein of the microvessel-enriched fractions of the hypothalamus and hippocampus.

In the DG, none of the modifications induced by phthalate exposure on BBB permeability and amounts of proteins involved in endothelial TJs and Cav-1 were observed, indicating that BBB integrity was preserved in this hippocampal vascular network. These results suggest that, within the same brain structure, the BBB may exhibit differential sensitivity to exposure to DEHP alone or in a mixture. This difference in sensitivity within the hippocampus suggests that maintaining BBB would involve different signalling pathways, with the DG containing neural circuits different from those of the CA1 and CA3 subregions and being the site of adult neurogenesis.

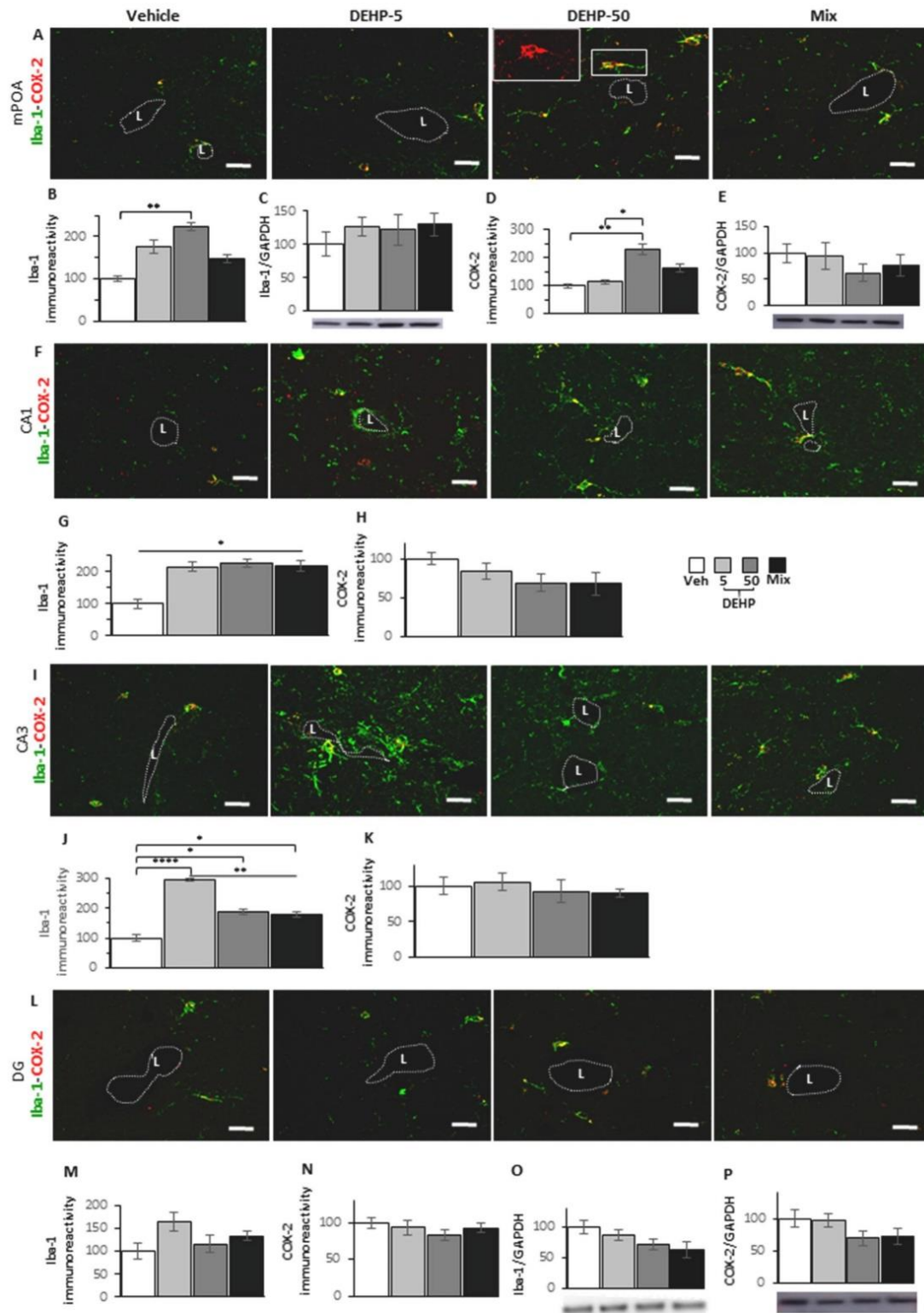
The modifications induced by exposure to DEHP alone or in a phthalate mixture in the BBB integrity were associated with astrocyte and microglial activation in the hypothalamic mPOA. Astrocyte activation was characterized by the increased immunoreactivity for both GFAP, NDRG2 and S100 β in the parenchyma of the mPOA. Firstly, this is in accordance with our recent study reporting that chronic adult exposure to DEHP at 50 $\mu\text{g/kg/d}$ induced an up-regulation of GFAP in the parenchyma of the mPOA in male mice (Dombret et al., 2017). Secondly, the data also indicate that adult exposure of male mice to DEHP alone or in a phthalate mixture was capable of affecting different steps of cellular activation and states. NDRG2 is induced in the early phase of astrocyte activation preceding GFAP expression (Lin et al., 2015), while S100 β is defined as a state in which astrocytes lose their neural stem cell potential and acquire a more mature developmental stage (Raponi et al., 2007). Western blot analysis performed with hypothalamic microvessel-enriched fractions showed a decreased GFAP amount in astrocyte end-feet associated with microvessels. This difference in the data protein obtained between the two techniques suggests a reorganization of the intermediate filament cytoskeleton of astrocytes involved in the BBB. Reactive astrocytes are known to express the pro-inflammatory molecule iNOS in response to various stimuli to produce an excessive amount of nitric oxide (NO), a gaseous signalling molecule involved in most cases with a neurodegenerative and neuro-inflammatory status (Saha and Pahan, 2006). A Western blot performed on microvessel-enriched fractions from the whole hypothalamus showed no changes in the iNOS protein levels, but higher iNOS labelling was colocalized with GFAP labelling astrocyte end-feet associated with capillaries in the mPOA. This suggests that the inflammatory reaction mediated by astrocytes was initiated at, or limited to, the level of the BBB. The astroglial activation in the parenchyma of the mPOA induced by the exposure to DEHP at 50 $\mu\text{g/kg/d}$ was accompanied by an activation of microglial cells, which also exhibited a higher immunoreactivity of the inflammatory molecule COX-2. The association between COX-2 expression and microglial activation could lead to NVU impairment through neuronal damage as has been previously reported

(Vijitruth et al., 2006). Further studies will compare the impact of phthalate exposure on neuronal integrity.

In the hippocampus, no changes in astrocyte activation markers were induced by adult exposure to DEHP alone or in a phthalate mixture, suggesting an absence of astrocyte activation in this region despite the increased BBB permeability. In contrast, microglial activation was observed in the CA1 and CA3 sub-regions following exposure to DEHP alone or in a phthalate mixture, whereas no changes in COX-2 immunoreactivity were demonstrated. A recent investigation has shown that prenatal exposure to DEHP at 200 $\mu\text{g/kg/d}$ increased the level of COX-2 in pyramidal neurons of the CA-2/3 region in adult male mice (Barakat et al., 2018). This suggests that differences in induced COX-2 expression by phthalates may depend on the period of exposure. Alternatively, a longer exposure period in adult males may be a more efficient way of inducing astrocyte activation and COX2 expression. Nevertheless, exposure to DEHP alone or in a mixture-induced iNOS activation was noted in the CA1 and DG sub-regions but not in the CA3 area, while BBB leakage was detected in both the CA1 and CA3 areas. The iNOS labelling was thus not colocalized with the astrocytic marker GFAP, suggesting that astrocytes of the BBB may not be the only cells capable of producing NO under these conditions. Indeed, different brain cell types including neurons, macrophages and microglia, can respond to different stimuli with iNOS production and take part in brain inflammation (Heneka and Feinstein, 2001; Sierra et al., 2014). It is thus possible that an increased amount of iNOS protein in the hippocampus may arise from an additional direct impact of DEHP alone or in a mixture on the parenchyma. Phthalates are able to cross the BBB thus eliciting direct effects through neuronal damage, neuroinflammation, oxidative stress and alteration of monoaminergic, cholinergic and amino-acids transmission as has been reported for adult or developmental exposure to DEHP or DBP at high doses ranging from 30 to 750 mg/kg/d (Kassab et al., 2019; You et al., 2018). Thus, the question remains as to whether the BBB dysfunction is a cause or a consequence of a neuroinflammatory state.

Altogether, these data show that the BBB dysfunction and generally associated astroglial activation and neuroinflammation were differently induced by adult exposure to DEHP alone or in a phthalate mixture (Table 2). Differences were seen between the hypothalamic mPOA and hippocampus, in particular for the astrocyte activation. Differences in sensitivity to exposure were also suggested by the obtained data for each brain area. In the mPOA, while BBB permeability and iNOS induction were affected since 5 $\mu\text{g/kg/d}$ of DEHP alone or in a mixture, the other parameters were rather altered at 50 $\mu\text{g/kg/d}$ of DEHP and/or the mixture, suggesting that the other phthalates add to the effects of DEHP-5. In the hippocampus, the DG appeared less sensitive showing only an induced iNOS in response to DEHP treatment while the CA1 and CA3 areas were more reactive, with effects induced since 5 $\mu\text{g/kg/d}$ of DEHP alone for BBB permeability, microglial activation and neuroinflammation.

The down-regulation of the androgen receptor (AR) induced by exposure to DEHP in the hypothalamic mPOA has been previously shown (Dombret et al., 2017). Thus, the alterations triggered by adult exposure to DEHP alone or in a phthalate mixture in the neurovascular unit of the mPOA may be also linked, at least in part, to the down-regulation of the AR. Furthermore, in another study, we have shown that testosterone depletion in adult male mice, leading to a



(caption on next page)

Fig. 6. Exposure to DEHP alone or in a phthalate mixture induced microglia activation in the testosterone-sensitive cerebral regions, the medial preoptic area (mPOA) and the hippocampus, and increased COX-2 expression in the mPOA. (A, B, D) Representative images (A) and corresponding quantitative analysis (B, D) of the six to eight serial sections for each brain examined of the co-immunolabeling of Iba-1 (green) and COX-2 (red, insert) in the mPOA of mice exposed to the vehicle (Veh), DEHP at 5 µg/kg/d (DEHP-5), DEHP at 50 µg/kg/d, or phthalate mixture (Mix) (n = 5 per treatment group). **p* < 0.05; ***p* < 0.01 compared to the vehicle group. (C, E) Western blot analysis (n = 6 per treatment group) of Iba-1 (C) and COX-2 (E) performed on microvessel-enriched fractions from hypothalamus. **p* < 0.05; ***p* < 0.01 compared to the vehicle group. Data were normalized to GAPDH level. (F–N) Representative images of co-immunodetection of Iba-1 (green) and COX-2 (red) in the hippocampus (F: CA1; I: CA3; L: DG) and their corresponding quantitative analysis of the immunoreactivity density (G, H: CA1; J, K: CA3; M, N: DG) of the six to eight serial sections for each brain examined (n = 5 per treatment group). **p* < 0.05; ***p* < 0.01; ****p* < 0.001; *****p* < 0.0001 compared to vehicle-treated controls. (O, P) Western blot analysis (n = 6 per treatment group) of Iba-1 (O) and COX-2 (P) performed on microvessel-enriched fractions from hippocampus. Data were normalized to GAPDH level. No significant difference was measured compared to vehicle-treated controls. Scale bar: 10 µm. L: Lumen delimited by the dotted lines. The quantifications of fluorescence density were measured over the entire surface of the images. All values represent mean percentages ± S.E.M of the vehicle (100%). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 2

Synthesis of the effects of an oral exposure during adulthood to low doses of DEHP alone or in phthalate mixture in male mice on BBB integrity and associated inflammation in the hypothalamus and hippocampus.

			Immunohistochemistry analysis				Western blot analysis	
			mPOA	CA1	CA3	DG	Hypothalamus	Hippocampus
BBB integrity	Permeability	Evans blue extravasation	increased: DEHP-5, DEHP-50 and Mix	increased: DEHP-5 and DEHP-50	increased: DEHP-50	not affected	n.d.	n.d.
		Endogenous IgG extravasation	increased: DEHP-5, DEHP-50 and Mix	increased: DEHP-5, DEHP-50 and Mix	increased: DEHP-5, DEHP-50 and Mix	not affected	n.d.	n.d.
	Endothelial tight junctions	Claudin-5	not affected	not affected	not affected	not affected	not affected	not affected
		Occludin	not affected	not affected	not affected	not affected	not affected	not affected
	Trans-endothelial vesicular transport	ZO-1	not affected	not affected	not affected	not affected	not affected	not affected
		Cav-1	decreased: DEHP-50 and Mix	decreased: DEHP-50 and Mix	decreased: DEHP-50 and Mix	not affected	not affected	not affected
Inflammation	Astrocyte activation	NDRG2	increased: Mix	not affected	not affected	not affected	n.d.	n.d.
		S100 p	increased: DEHP-50 and Mix	not affected	not affected	not affected	n.d.	n.d.
	Microglia activation	GFAP	increased: DEHP-50	not affected	not affected	not affected	decreased: DEHP-5, DEHP-50 and Mix	not affected
		Iba-1	increased: DEHP-50	increased: DEHP-5, DEHP-50 and Mix	increased: DEHP-5, DEHP-50 and Mix	not affected	not affected	not affected
	Inflammatory molecules	iNOS	increased: DEHP-5, DEHP-50 and Mix	increased: DEHP-5, DEHP-50 and Mix	not affected	increased: DEHP-5	not affected	not affected
		COX-2	increased: DEHP-50	not affected	not affected	not affected	not affected	not affected

n.d.: not determined; DEHP-5: DEHP at 5 µg/kg/d; DEHP-50: DEHP at 50 µg/kg/d; Mix: phthalate mixture.

down-regulation of the neural AR, triggered i) BBB permeability for Evans Blue and endogenous IgG with a disorganization of TJ structure including a reduced protein amount of ZO-1, ii) increased activation of astrocytes and microglia and iii) up-regulation of inflammatory molecules such as iNOS and COX2 (Atallah et al., 2017). Finally, the AR mediated-transcriptional activity is, at least in part, positively modulated by the caveolae transmembrane Cav-1 protein (Bennett et al., 2010; Lu et al., 2001). The tight regulation by androgens of several proteins involved in these processes is detailed in a recent review (Ahmadpour and Grange-Messent, 2021).

The hippocampus is also an androgen-sensitive area, as evidenced by a high AR expression in male mice and altered related functions in a mouse model lacking the neural AR (Mhaouty-Kodja, 2018; Picot et al., 2016; Raskin et al., 2009). It is thus possible that the alterations reported here in the neurovascular unit of this structure may also depend, at least in part, on an impact on the AR signalling pathway. This can explain the comparable data obtained on BBB permeability, endothelial TJs and trans-endothelial vesicular transport in the mPOA and CA1 and CA3 regions following exposure to DEHP alone or in a phthalate mixture (Table 2). The CA1 and CA3 regions contain much more AR-immunoreactive neurons than the DG (Raskin et al., 2009), which may explain the reduced sensitivity of this latter area to DEHP exposure.

The differences observed in astrocyte activation and COX-2 expression between the mPOA and the hippocampal CA1 and CA3 regions, and also within the same brain area for the different components of the neurovascular unit could be underlined by different expression patterns of AR but also of estrogen receptors (ERs) in neurons, endothelial cells, astrocytes and microglia, which also express these receptors (Hajszan et al., 2007; Kerr et al., 1995; Picot et al., 2016). Indeed, in the male rodent nervous system, testosterone exerts its effects through direct activation of the AR, but can also be metabolized into estradiol, which then stimulates ERα and ERβ.

The effects induced by DEHP exposure i.e. BBB permeability, astroglial activation and neuroinflammation in adult male mice may participate, in particular in the mPOA, to the altered sexual behaviour observed under similar experimental conditions (Dombret et al., 2017). Current studies explore the cognitive impact of male mice exposure to low doses of phthalate but only one recent study addressed the effects of adult DEHP exposure on spatial learning and memory at high doses of 100 and 300 mg/kg/d in male rats (Ran et al., 2019). Besides behavioural alterations, the BBB leakage and inflammatory signs induced by oral exposure to low doses of phthalates reported in the present study may participate in other neural alterations. Although they appear moderate, these modifications could have deleterious long-term

consequences in the case of chronic environmental exposure. Indeed, BBB impairment and neuroinflammation are suspected to be involved in neurodegenerative diseases and aging processes (Erdő et al., 2017).

Our analyses addressing the impact of DEHP exposure on behavioural responses (Dombret et al., 2017) and BBB integrity (present study) in male mice show that DEHP-induced effects can be observed at low doses equivalent or below the tolerable daily intake dose of 50 µg/kg/d, which was established and recently updated by the European Food Safety Authority on the basis of reduced fetal testosterone production. This indicates that the nervous system including the neurovascular unit is highly sensitive to phthalates and should be also considered as a relevant endpoint in risk assessment for these molecules. In this context, it is important to stress that the human cerebrovascular function also seems sensitive to androgen, although these effects are less defined than for estrogens (see for review (Robison et al., 2019)).

5. Conclusion

The present study shows that phthalates, a family of endocrine disruptors, can impact the cerebrovascular function at doses close to the environmental exposure in adult male mice. Subchronic exposure to low doses of DEHP alone or in a phthalate mixture increases BBB permeability of two brain regions leading to a dysfunctionality of this selective interface, which is needed to ensure a tight regulation between the circulatory system, the immune system and the brain parenchyma. In addition, we also show similarities and differences in the vulnerability of the NVU of the two brain regions, one controlling male sexual behaviour, and the second one involved in cognitive function. Based on our previous studies, we suggest that phthalates may operate through a mode of action involving at least a partial disruption of neural AR expression in the mPOA, but the ER and/or other signalling pathways may be also involved in the hippocampus.

These data suggest that exposure to endocrine disruptors may be considered as an environmental risk factor for the cerebrovascular function.

Credit author statement

Delnia Ahmadpour: Investigation, Formal analysis, Writing – original draft. Valérie Grange-Messent: Conceptualization, Supervision, Writing – review & editing. Sakina Mhaouty-Kodja: Funding acquisition, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This work was supported by the Agence Nationale de la Recherche, France (Phtailure, 2018). We thank the rodent facility of the Institut de Biologie Paris-Seine (IBPS, Paris, France) for taking care of the animals and the IBPS Imaging facility (confocal microscopy).

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.chemosphere.2021.131013>.

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supplementary data

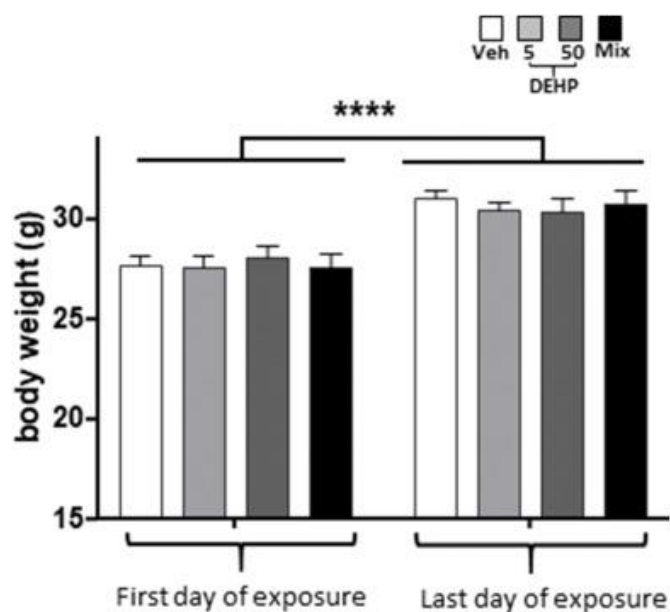
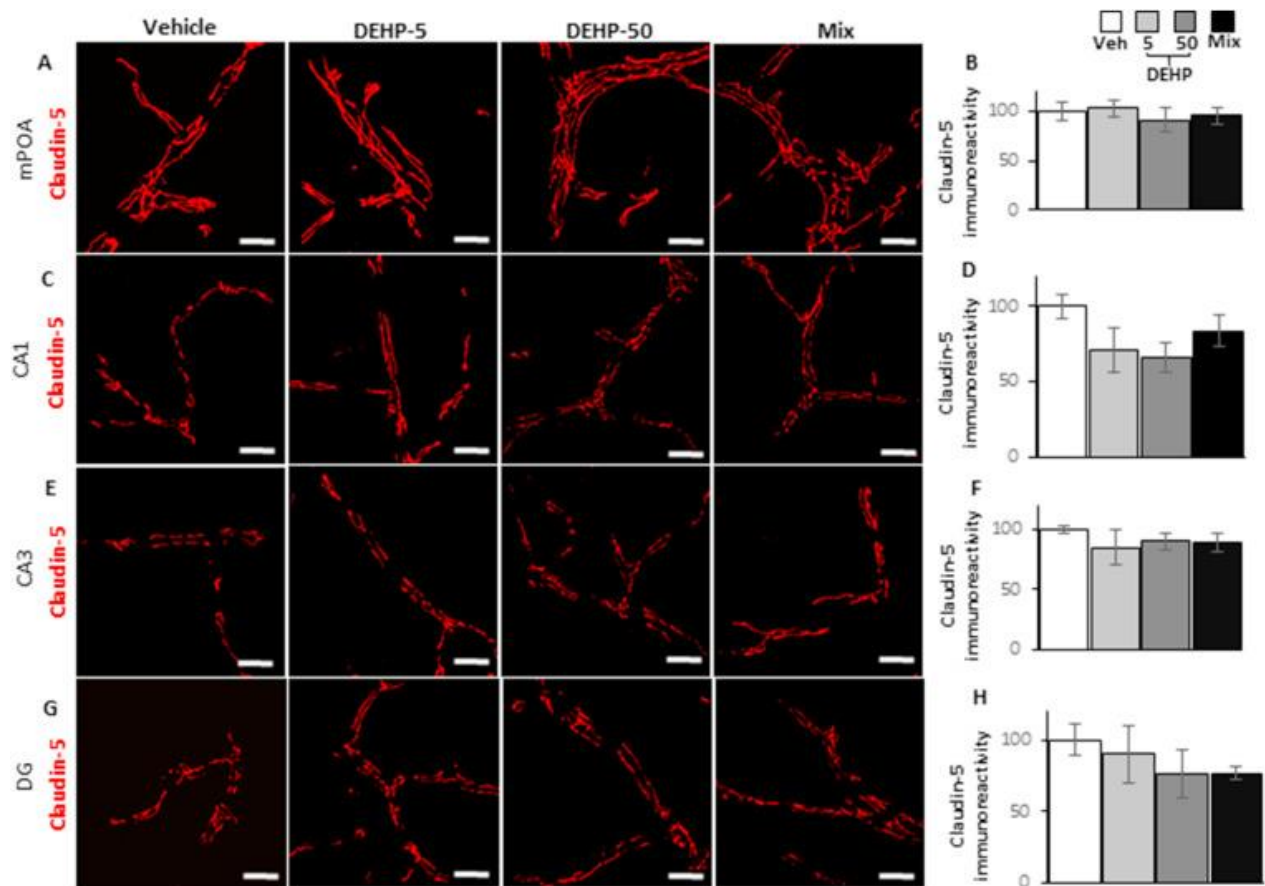
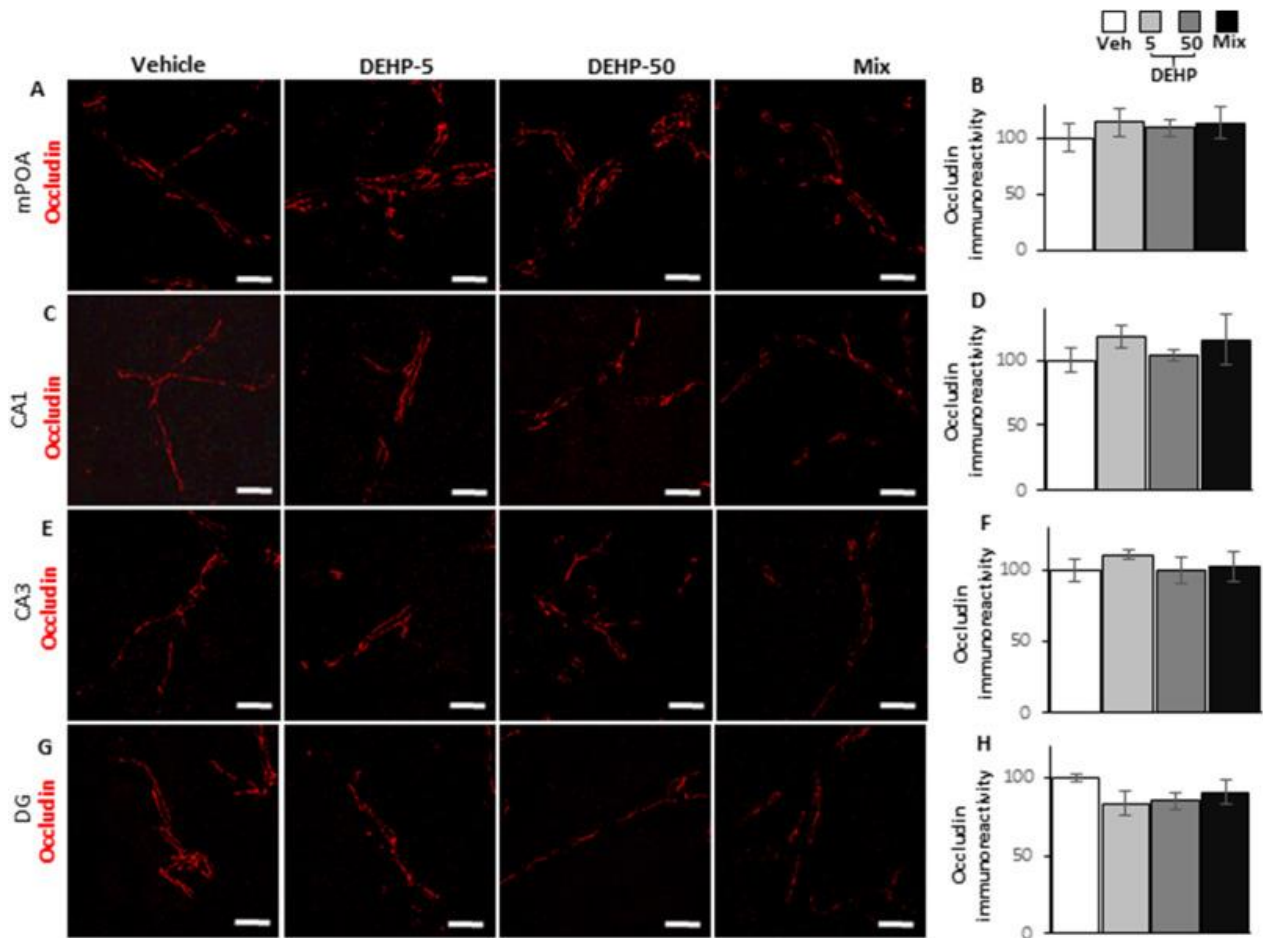


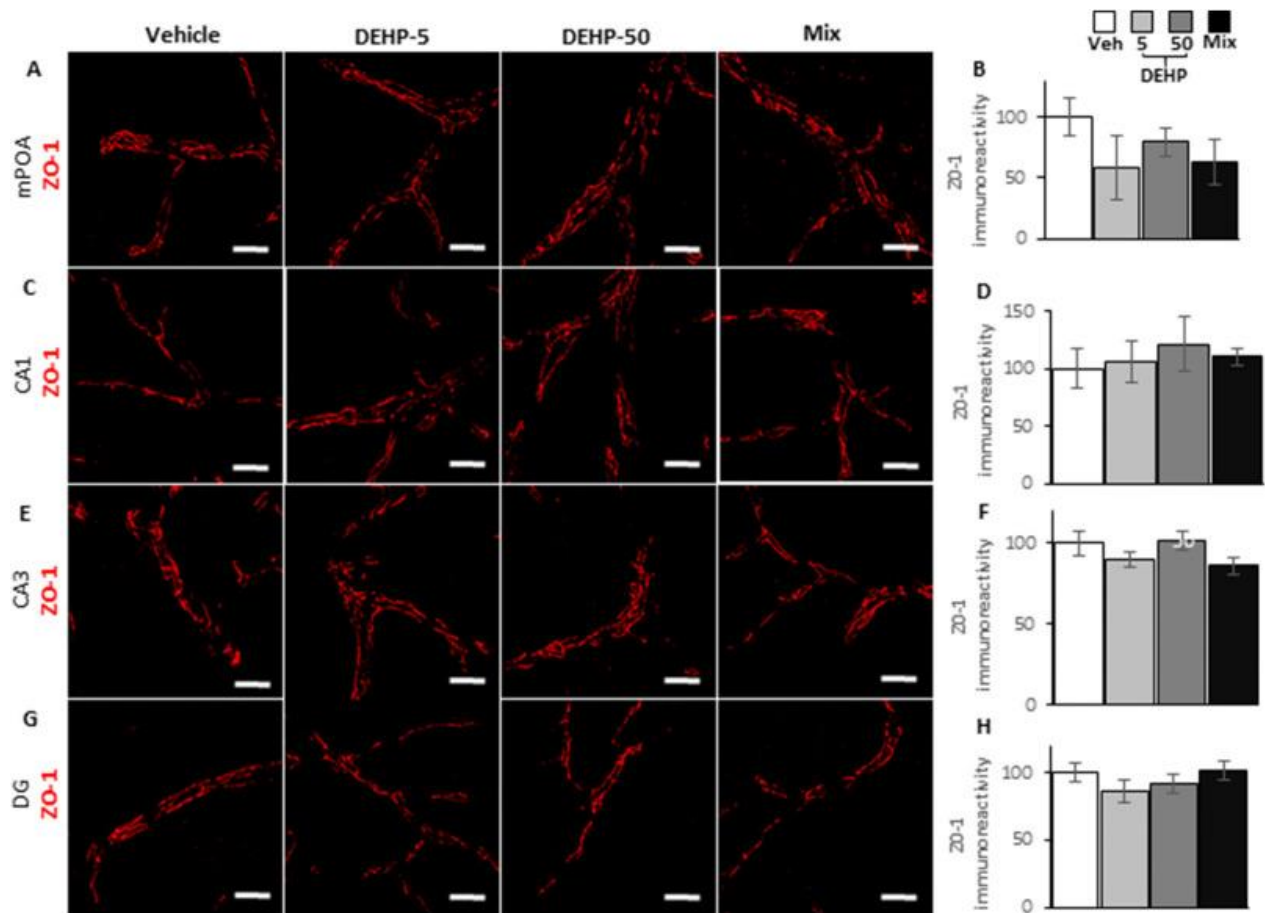
Figure S1. Exposure to DEHP alone or in a phthalate mixture did not affect the body weight of treated mice. The body weight of male mice exposed to the vehicle (Veh), DEHP at 5 $\mu\text{g/kg/d}$ (DEHP-5), DEHP at 50 $\mu\text{g/kg/d}$ (DEHP-50), or a phthalate mixture (Mix), is reported for the first and last days of exposure. The values represent body weight means (g) \pm S.E.M (n = 12 per treatment group). There was an effect of time ($F_{(1, 88)} = 48.8, p < 0.0001$) but not of treatment ($F_{(3, 88)} = 0.1063, p = 0.9562$).



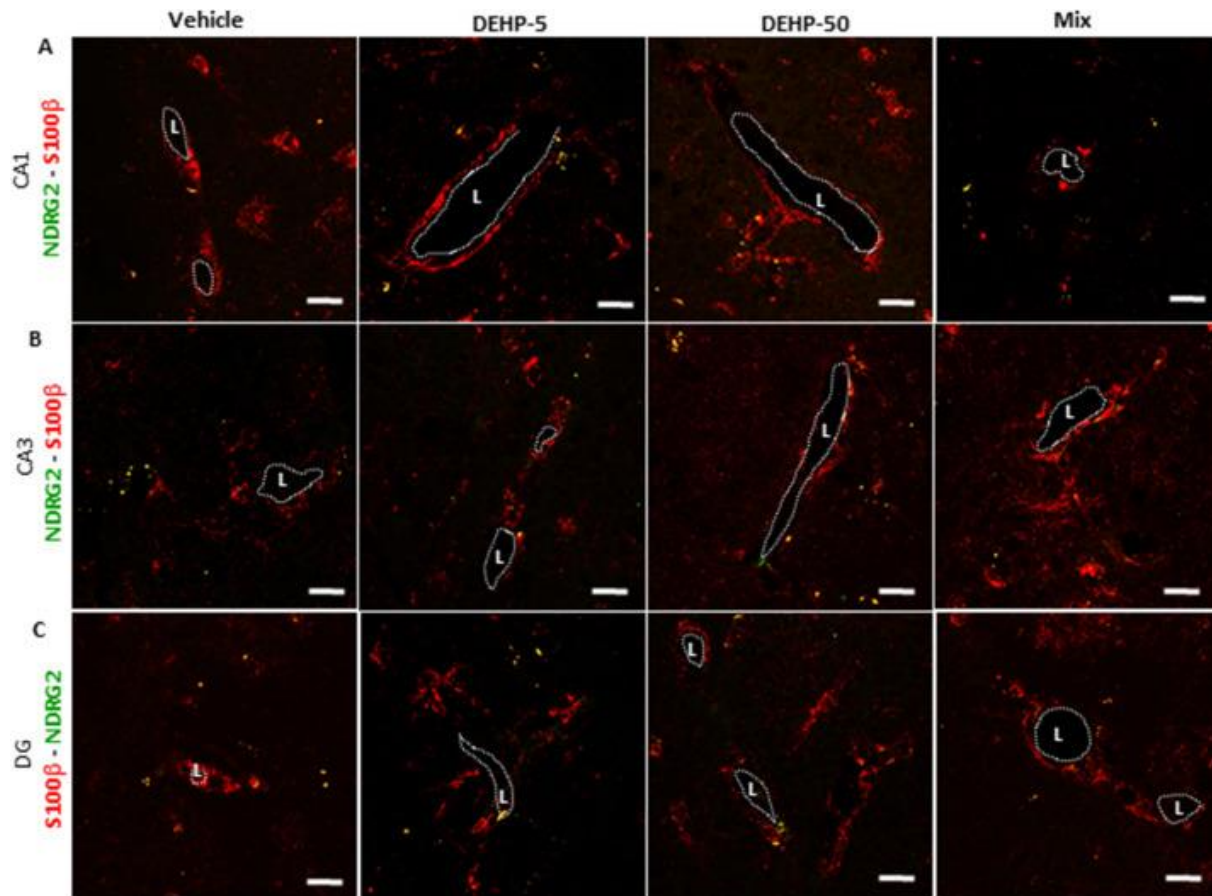
Color Figure S2. Exposure to DEHP alone or in a phthalate mixture did not affect claudin-5 immunoreactivity. (A, B) Representative images of immunodetection of claudin-5 in the mPOA of mice exposed to the vehicle (Veh), DEHP at 5 $\mu\text{g/kg/d}$ (DEHP-5), DEHP at 50 $\mu\text{g/kg/d}$ (DEHP-50), or phthalate mixture (Mix) (A) and their corresponding quantitative analysis of the immunoreactivity density (B) of the six to eight serial sections for each brain examined ($n = 5$ per treatment group). No significant difference was measured compared to vehicle-treated controls. (C-H) Representative images of immunodetection of claudin-5 (C: CA1; E: CA3; G: DG) and the corresponding quantitative analysis of the immunoreactivity density (D: CA1; F: CA3; H: DG) of the six to eight serial sections for each brain examined ($n=5$ per group). No significant difference was measured compared to vehicle-treated controls. Scale bar: 10 μm . The quantifications of fluorescence density were measured over the entire surface of the images and the values represent mean percentages \pm S.E.M of vehicle (100%).



Color Figure S3. Exposure to DEHP alone or in a phthalate mixture did not affect occludin immunoreactivity. (A, B) Representative images of immunodetection of occludin in the mPOA of mice exposed to the vehicle (Veh), DEHP at 5 $\mu\text{g/kg/d}$ (DEHP-5), DEHP at 50 $\mu\text{g/kg/d}$ (DEHP-50), or phthalate mixture (Mix) (A) and their corresponding quantitative analysis of the immunoreactivity density (B) of the six to eight serial sections for each brain examined ($n=5$ per group). No significant difference was measured compared to vehicle-treated controls. (C-H) Representative images of immunodetection of occludin in the hippocampus (C: CA1; E: CA3; G: DG) and the corresponding quantitative analysis of the immunoreactivity density (D: CA1; F: CA3; H: DG) of the six to eight serial sections for each brain examined ($n = 5$ per treatment group). No significant difference was measured compared to vehicle-treated controls. Scale bar: 10 μm . The quantifications of fluorescence density were measured over the entire surface of the images and the values represent mean percentages \pm S.E.M of vehicle (100%).



Color Figure S4. Exposure to DEHP alone or in a phthalate mixture did not affect ZO-1 immunoreactivity. (A, B) Representative images of immunodetection of ZO-1 in the mPOA of mice exposed to the vehicle (Veh), DEHP at 5 $\mu\text{g/kg/d}$ (DEHP-5), DEHP at 50 $\mu\text{g/kg/d}$ (DEHP-50), or a phthalate mixture (Mix) (A) and their corresponding quantitative analysis of the immunoreactivity density (B) of the six to eight serial sections for each brain examined ($n=5$ per group). No significant difference was measured compared to vehicle-treated controls. (C-H) Representative images of immunodetection of ZO-1 in the hippocampus (C: CA1; E: CA3; G: DG) and the corresponding quantitative analysis of the immunoreactivity density (D: CA1; F: CA3; H: DG) of the six to eight serial sections for each brain examined ($n = 5$ per treatment group). No significant difference was measured compared to vehicle-treated controls. Scale bar: 10 μm . The quantifications of fluorescence density were measured over the entire surface of the images and the values represent mean percentages \pm S.E.M of vehicle (100%).



Color Figure S5. Exposure to DEHP alone or in a phthalate mixture did not affect NDRG2 and S100β immunoreactivity in the hippocampus. Representative images of co-immunodetection of NDRG2 (green) and S100β (red) in the hippocampal CA1 (A), CA3 (B) and DG (C) of mice exposed to the vehicle (Veh), DEHP at 5 µg/kg/d (DEHP-5), DEHP at 50 µg/kg/d (DEHP-50), or phthalate mixture (Mix). Scale bar: 10 µm. L: Lumen delimited by the dotted lines.

5.2 Part B: Androgen receptor-mediated cellular pathway underlying the hypothalamus neurovascular unit impairment, following adult exposure to low doses of di(2-ethylhexyl) phthalate alone or in an environmental phthalate mixture in male mice.

Phthalates are endocrine disruptor chemicals showing effects on endocrine systems including those involved in the control of the male reproductive functions by displaying an anti androgenic activity. Our team has reported that chronic low-dose exposure to DEHP during adulthood disrupts the emission of courtship vocalizations and initiation of mating in male mice (Dombret et al., 2017). This alteration is not associated with modifications of circulating testosterone levels and/or HPG axis, but with a reduced androgen sensitivity confirmed by the demonstration of AR down-regulation in the median preoptic area (mPOA). Sexual behavior is triggered in males following the integration of chemosensory signals at the level of the mPOA under the control of sex steroid hormones. Thus, the mPOA is a highly sensitive brain area to gonadal testosterone, involved in the central control of male reproduction.

Male cerebral vessels are target tissues of testosterone as they express androgen and estrogen receptors and enzymes involved in the biosynthesis of estradiol and dihydrotestosterone (Gonzalez et al., 2007, 2010). It has been shown that reduced androgen sensitivity through testosterone depletion in adult male mice induces glial activation and up-regulation of inflammatory molecules which are associated with an increased blood-brain barrier (BBB) leakage in cerebral capillaries of the mPOA (Atallah et al., 2017). The BBB acts as a selective and highly regulated interface between the circulatory system and the brain parenchyma. It is formed by the brain endothelial cells at the level of the brain capillary. BBB is formed by tightly bound endothelial cells and is an essential part of the neurovascular unit (NVU), a complex anatomical and functional multicellular structure comprised of a basement membrane covered with pericytes, neurons, glia cells including astrocytes end feet and microglial.

Recently we have revealed that subchronic exposure of adult male mice to low doses of DEHP, alone or in an environmental phthalate mixture, led to an impairment of the BBB in the

brain region sensitive to testosterone such as the hypothalamic mPOA. We have demonstrated that exposure to a tolerable daily intake (TDI) dose (50 µg/kg/day), or to the environmental dose (5 µg/kg/day) of DEHP alone or in a relevant mixture containing the most commonly phthalates detected in the environment, induced a BBB leakage. Increased BBB permeability was due to an alteration of the junctional accessory protein Zonula Occludens (ZO-1) and decrease of the main component of caveolae-transmembrane protein, caveolin 1 (Cav-1). The alteration of the BBB was also associated with glial activation affecting microglia and astrocytes, and an increase of iNOS in perivascular astrocyte end-feet (Ahmadpour et al., 2021). Then, we have suggested that failure of BBB functionality in the mPOA could participate, at least in part, to the reported-behavioral alteration following phthalates exposure (Ahmadpour et al., 2021).

The underlying mechanisms involved in the phthalates-induced alteration of molecular and cellular constitutive elements of the neurovascular unit in the mPOA are unknown. Therefore, it is of particular interest to investigate the molecular mechanisms involved in phthalates-induced NVU alteration. In this context, this present work explored the underlying mechanisms and continued to characterize the adverse effects of phthalate exposure.

The presence of AR and ERα in capillaries located in the mPOA of untreated adult male mice was first researched by electron microscope immunocytochemistry. In order to explore the anti-androgenic activity of phthalates at the level of the brain capillary, we studied the effects of exposure on AR et ERα protein amounts by Western blot protein analysis using hypothalamus capillary-enriched factions. Based on our previous data, we hypothesized that phthalates exposure could mediate an increase in matrix metalloproteinase (MMPs) expression and/or activity levels, thereby contributing to BBB leakage. Then, we investigated the effects of phthalate exposure on the gelatinases, MMP-2 and MMP-9, the key regulatory molecules involved in the regulation of all extracellular matrix components and the adhesion proteins which are involved in the cell-cell and cell-matrix interactions by both *in situ* Zymography in the mPOA and Western blot analyses performed on capillary-enriched fractions. The integrity of the basement membrane and adhesion proteins dedicated to cell-cell and cell-matrix interactions, was also investigated using immunohistochemistry and Western blot analyses. In parallel, the effect of exposure on

neurodegenerative process using Fluoro-Jade® C staining and ultrastructural observation was examined.

Analyses were processed using four experimental groups of adult male mice exposed orally through contaminated diet in order to mimic the major route of exposure: two groups of animals exposed to DEHP alone at low doses (5 µg/kg/day and 50 µg/kg/day), one group of animals exposed to DEHP (5µg/kg/day) in combination with other phthalates abundant in the environment, and one control group of animals exposed to vehicle. We focused on the highly sensitive brain area with respect to gonadal testosterone, hypothalamic mPOA, in which we have already shown a NVU dysfunction following the above mentioned- phthalate exposure doses.

The presence of AR and ERα receptors was evidenced, at least partly, within endothelial cells of capillaries in adult male mice, free in cytoplasm or closely associated with plasma membrane or organelles such as mitochondria, suggesting they could also be targets for anti-androgenic endocrine disruptors. Exposure to DEHP at 50 µg / kg / day or at 5 µg / kg / day in a phthalate mixture, induced a decrease of the expression of the AR but not ERα protein. These data allow to suggest a possible involvement of the AR non-genomic signaling pathway which could lead to a rapid vascular response to androgen-signaling disruptor compounds in this testosterone-sensitive brain area. AR interact with a large number of co-regulators which function as transactivation chaperones, such as the caveolin-1 isoform protein (Cav-1). Transient interaction between AR and Cav-1 may be necessary prior to non-genomic AR activity originating from caveolae structures.

Higher levels of gelatinolytic activity of MMP-2 and MMP-9 in the mPOA after phthalate treatment compared to vehicle, without any treatment effect on their protein amounts, were observed. This increased activity of MMPs could be due, at least in part, to the decrease in Cav-1 protein levels leading to a disinhibition of NOS and an increase of the production of NO. This enhanced gelatinolytic activity could mediate the rearrangement and/or degradation of ZO-1, leading subsequently to the BBB leakage previously observed.

Exposure to DEHP alone or in an environmental phthalate mixture induced a decrease of laminin α -1, β -dystroglycan and collagen IV. Thus, the increase of the gelatinase activity following phthalates exposure may lead, to an alteration / remodeling of the ECM surrounding brain capillaries which could be also responsible of the neurovascular unit impairment in this hypothalamic brain area. In addition, such exposure to phthalates led to neurodegenerative process in this brain area. Astrocyte activation, leading to inflammatory mediator production, and MMP activation, leading to the neurovascular unit impairment, could be responsible to the present observed neuronal degenerative process in the mPOA.

A decrease of interaction between AR and Cav-1 protein in brain capillaries, a consequence of their level decrease induced by phthalates, leads to the activation of gelatinases which, in turn, generates alteration of the basement membrane and of cell-matrix interactions, resulting in a disruption of the neurovascular unit and neuronal damages. This lesser AR / Cav-1 interaction would also lead to an excessive amount of NO produced following phthalate-induced disinhibition of iNOS in astrocyte end-feet associated with capillaries. NO is a signalling free radical involved, among others, in most pathologic features associated with a neurodegenerative and neuroinflammatory status

This proposed androgen receptor-mediated cellular pathway, would lead to a failure of the glio-neuro-vascular coupling and NVU integrity which could be involved in the impairment of the male sexual behavior (**Fig.40**).

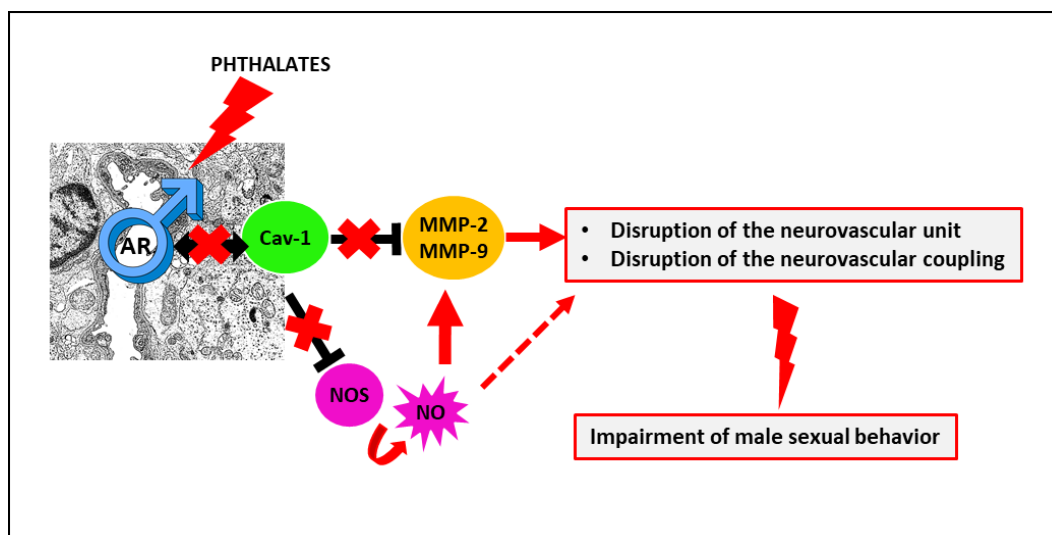


Figure 40. Proposed cellular pathway for glio-neurovascular coupling impairment following adult exposure to low doses of DEHP alone or in an environmental phthalate mixture in male mice

As described above, a decrease of interaction between AR and Cav-1 protein in brain capillaries, a consequence of phthalates exposure, leads to the activation of gelatinases which, in turn, generate alteration of the basement membrane and of cell-matrix interactions. This could result in a disruption of the glio-neurovascular unit and neuronal damages. This lesser AR / Cav-1 interaction would also lead to an excessive amount of NO produced following phthalate-induced disinhibition of iNOS in astrocyte end-feet associated with capillaries. This proposed androgen receptor-mediated cellular pathway could be disrupted in the phthalate-induced impairment of the male sexual behavior

To note that, all mentioned experiments were also performed on the different subregions of the hippocampus (CA1, CA3 and DG). As previously mentioned, hippocampus is an essential part of the limbic system, responsible for cognition function and emotional responses. In male, Hippocampus exhibits a high sensitivity to testosterone and high vulnerability to phthalate exposure (Ahmadpour et al, 2021). However, despite the fact that phthalates induce NVU alteration in both regions, as our result highlighted (data not shown), phthalate exposure does not act in a similar way in those regions. In this context, we will deepen our research on deciphering the underlying mechanism induce NVU impairment following phthalates exposure in the hippocampus. Such investigations will shed light on phthalates-induced mechanisms of cognition alteration and anxiety- and depressive-like behavior (Barakat et al., 2018; Xu et al., 2015b).

Article

Deciphering of an androgen receptor-mediated cellular pathway involved in the hypothalamus neurovascular unit impairment following adult exposure to low doses of di(2-ethylhexyl) phthalate alone or in an environmental phthalate mixture in male mice

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Key words: androgen receptor, endocrine disruptors, hypothalamus, neurovascular unit, phthalates

Abstract

We have previously shown that adult male mice exposure to low doses of di(2-ethylhexyl)phthalate (DEHP) impacts the blood-brain barrier (BBB) integrity and surrounding parenchyma in the hypothalamus medial preoptic area (mPOA). The BBB leakage was associated with a decrease of endothelial tight junction accessory protein, zona occludens-1 (ZO-1), and caveolae protein Cav-1, added with an inflammatory profile including glial activation accompanied by enhanced expression of inducible nitric oxide synthase. Adult exposure to low doses of DEHP alone or in an environmental phthalate mixture also disrupts the initiation of mating in male mice. This failure of BBB functionality in the mPOA could participate, at least in part, in the reported alteration of sexual behavior, but the cellular pathway connecting cerebral

capillaries and neurons remained to be explored. Two-month-old C57BL/6J males were orally exposed for 6 weeks to DEHP alone (5 and 50 µg/kg/day) or to DEHP (5 µg/kg/day) in an environmental phthalate mixture. The presence of androgen receptor (AR) and estrogen receptor-α (ERα) was first evidenced in brain capillaries. AR protein levels, without change of ERα protein levels, was decreased in cerebral capillaries after phthalate exposure. Basement membrane and cell-matrix interactions components were decreased, while matrix metalloproteases MMP-2 and MMP-9 activity was increased. Such exposure to phthalates leads to neurodegenerative process in this brain area. The data highlight that environmental exposure to endocrine disruptors such as phthalates, could alter AR / Cav-1 interaction, impacting a Cav-1 / nitric oxide / MMP pathway, thus leading to disruption of essential glio-neurovascular coupling to the functioning of neurons.

Introduction

The blood-brain barrier (BBB) is a highly regulated specific interface at the level of cerebral capillaries that maintains homeostasis of the central nervous system (CNS) to ensure neuronal activity underlying the regulation of all physiological functions (Daneman and Prat, 2015). BBB allows the control of substance delivery from the blood compartment to the brain parenchyma, as well as the elimination of compounds from the parenchyma to the blood flow. Cerebral endothelial cells strictly limit the passage across the BBB through inter-endothelial tight junction systems (TJs), selective transporters expressed on the luminal/abluminal side of endothelial cells as well as low rate of caveolae-mediated transcellular vesicular transport (Abbott et al., 2010). ECs are surrounded by a basement membrane, which is a unique form of the extracellular matrix (ECM). Basement membrane also embedding pericytes interacts with astrocytic end-feet (Correale and Villa, 2009), which dynamic nature depends on its constitutive remodeling by several protease families, including the matrix metalloproteases (MMPs) (Rempe et al., 2016). The whole is called the neurovascular unit (NVU) and its integrity depends on the proper functioning of each element of this complex structure.

Cerebral vessels are target tissues for gonadal sex steroid hormones (Gonzales et al., 2007). In male, testosterone is the main sex steroid hormone and activates not only androgen receptor (AR) but also estrogen receptor α (ER α) following neural aromatization of testosterone into estradiol (see for review, Mhaouty-Kodja, 2018). Testosterone was shown not only to promote cerebral angiogenesis and vasculature formation, but also to modulate the cerebrovascular function (see for review, Ahmadpour and Grange-Messent, 2020), such as the integrity and function of the BBB, and could prevent gliosis reaction and up-regulation of inflammatory components in male mice (Atallah et al., 2017). Anti-androgenic compounds such as phthalates are known impairing the endocrine system function leading to adverse health effects (Benjamin et al., 2017; Dombret et al., 2017; Zlatnik, 2016). These endocrine disruptors are high production volume chemicals used as a plasticizer in plastic-based consumer products. Due to their extensive distribution in several daily life applications, they are widely propagated in our environment (Gao and Wen, 2016). Di(2-ethylhexyl) phthalate (DEHP) is the major detected phthalate family compound in the environment and has been classified since 2000 by the European Union as a priority substance “presenting a significant risk to or via the aquatic environment” in the Water Framework Directive 2000/60/EC, which was updated in 2008 and 2013 (Directive 2013/39/EU of the European Parliament and of the Council of 12 August 2013). In addition to DEHP, other phthalates including diethyl phthalate (DEP), dibutyl phthalate (DBP), butyl benzyl phthalate (BBP), and diisobutyl phthalate (DiBP) are also commonly detected in the environment (Gao and Wen, 2016). Phthalates were mainly studied for their adverse effects on reproductive function in human or wild life (see for review, Mhaouty-Kodja et al., 2018). However, the link between phthalates exposure and CNS function impairment has begun to emerge. Majority of *in vivo* studies have focused on the effects of perinatal exposure but the effects of an adult exposure to low doses of phthalates are still largely under-explored.

We have recently shown in a previous work that low doses of DEHP, alone or in an environmental phthalate mixture, have adverse effects on the NVU in adult male mice, which allowed us to suggest that exposure to endocrine disruptors may be considered as an environmental risk factor for the cerebrovascular function (Ahmadpour et al., 2021). Following an adult exposure at the

tolerable daily intake (TDI) dose of 50 µg/kg/day (European Food Safety Authority (EFSA) 2005, 2019), or at the dose close to environmental exposure of 5 µg/kg/day of DEHP alone or in a relevant mixture containing the most commonly phthalates detected in the environment listed above, a BBB leakage was observed in the hypothalamic medial preoptic area (mPOA), the main cerebral area involved in the expression of male sexual behavior. This BBB leakage was sustained by a decrease of the levels of the tight junction's accessory protein Zonula Occludens (ZO-1) and of the caveolin-1 isoform protein (Cav-1), the main component of caveolae-transmembrane protein. These features were accompanied by a glial activation involving capillary-associated microglia and astrocytes (Ahmadpour et al., 2021). Moreover, such exposure to low doses of DEHP impairs sexual behavior of male mice through a down-regulation of neural AR expression in the mPOA without modification of circulating testosterone levels and/or HPG axis (Dombret et al., 2017). Then, we have suggested that failure of BBB functionality in the mPOA could participate, at least partly, to the reported-behavioral alteration following phthalates exposure (Ahmadpour et al., 2021).

Thus, our present work aims to explore what is the underlying cellular pathway which is damaged in those alterations of the NVU in the mPOA, induced by an adult exposition to low doses of phthalates.

To answer this question, experiments were performed as previously described, using four experimental groups of adult C57BL/6J male mice exposed orally through contaminated diet in order to mimic the major route of exposure as previously described (Adam et al., 2021; Ahmadpour et al., 2021). The first three groups included males exposed for 6 weeks to the vehicle (control), DEHP at the TDI dose of 50 µg/kg/d, or DEHP at 5 µg/kg/d. The DEHP dose of 5 µg/kg/d is within the environmental exposure range; this dose induced behavioral alterations in male mice following adult or pubertal exposure (Capela and Mhaouty-Kodja, 2021; Dombret et al., 2017). In order to mimic environmental co-exposure to phthalates (Anses, 2015; Dewalque et al., 2014; Martine et al., 2013), the fourth group of males was exposed to a phthalate mixture containing DEHP at 5 µg/kg/d, DBP at 0.5 µg/kg/d, BBP at 0.5 µg/kg/d, DiBP at 0.5 µg/kg/d and DEP at 0.25 µg/kg/d.

We first investigated the presence of AR and ER α in brain capillaries and more particularly into cerebral endothelial cell. Then, AR and ER α protein levels in cerebral capillaries were respectively compared after phthalates exposure. Proteolytic activity of matrix metalloproteases MMP-2 and MMP-9 and their respective protein levels were investigated. Basement membrane and cell-matrix interactions components were assessed in the four exposed groups. We also evaluated the effect of phthalates exposure on neurodegenerative process in this brain area.

Materials and methods

Ethical statement

The experiments have been reported in compliance with the Animal Research: Reporting in Vivo Experiments (ARRIVE) guidelines. All studies were performed in compliance with the National Institute of Health guidelines for the care and use of Laboratory Animals (NIH Guide) and French and European legal requirements (Decree 2010/63/UE). Experiments were performed accordingly, to minimize animal number and discomfort and were approved by the “Charles Darwin” Ethical committee (project number 01490-01).

Animals

Males of C57BL/6j strain (Janvier Labs, Le Genest-Saint-Isle, France) bred in our laboratory were housed in a conventional facility after weaning under controlled photoperiod (12:12h light dark cycle-lights on at 1 p.m.), maintained at 22°C and relative humidity (60 \pm 10%), and had free access to water and a standard diet (A03–10; Safe-diets, Augy, France). The mice were housed in nest-enriched polysulfone cages, with polysulfone bottles. Offspring were mixed at the weaning to avoid potential litter effects with no more than one male per litter per cage, and were allowed to grow to 8 weeks of age.

Phthalate exposure

Exposure to phthalates (Sigma Aldrich, Saint-Quentin Fallavier, France) was performed as already described (Adam et al., 2021; Ahmadpour et al., 2021). The phthalates, first dissolved in absolute

ethanol, were incorporated into food as previously detailed (Ahmadpour et al., 2021). Eight-week-old males were fed *ad libitum* for 6 weeks with chow reconstituted into pellets containing the vehicle i.e. ethanol and water (control group), DEHP (CAS 117-81-7) at 50 or 5 µg/kg/d (DEHP-50 and DEHP-5 groups, respectively), or a phthalate mixture (Mix group) containing DEHP at 5 µg/kg/d, DBP (CAS 84-74-2) at 0.5 µg/kg/d, BBP (CAS 85-68-7) at 0.5 µg/kg/d, DiBP (CAS 84-69-5) at 0.5 µg/kg/d and DEP (CAS 84-66-2) at 0.25 µg/kg/d. The composition of the phthalate mixture was based on French and European studies showing an external co-exposure to these molecules and the presence of their metabolites in urinary samples (Dewalque et al., 2014; Martine et al., 2013). Mice were weighed weekly for the duration of the exposure and phthalate doses were adjusted to their body weights and calculated for a daily food intake of 5 g per animal (Cheema et al., 2019; Dombret et al., 2017). It has already been shown that such a phthalate exposure protocol had no effect on the body weight of individuals between the first and the last day of treatment (Ahmadpour et al., 2021).

The analyses were performed on 4 cohorts each comprising animals distributed equally between the 4 treatment groups. Briefly, two cohorts were used for immunohistochemistry, immunocytochemistry and microscopy study. One was used for the capillary-enriched fraction procedure for western blot analysis and then one cohort was used for *in situ* zymography and Fluoro-Jade® C fluorescent investigation.

Electron microscope immunocytochemistry

Mice (n=3 per treatment group) were deeply anaesthetized with a lethal dose of pentobarbital (120 mg/kg, i.p.) and then transcardially perfused with 0.9% saline solution followed by a solution containing 0.2% glutaraldehyde, 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4. Brains were carefully removed, post-fixed with the same fixative solution overnight at 4°C and cut using a vibratome. Four to six floating frontal sections (40 µm thick) including the mPOA were treated by incubation in 0.1 M phosphate buffer saline (PBS) containing 1% bovine serum albumin (BSA) for 30 min at room temperature (RT) in order to block non-specific immune sites. Sex steroid receptors AR or ERα were detected using specific primary rabbit antibodies (Table 1) each diluted 1:200 in 0.1 M PBS-1% BSA, for 48 h at 4°C. After washes in PBS / BSA, immune complexes were

fixed using a solution containing 0.1% glutaraldehyde in 0.1 M PB, pH 7.4 for 10 min at RT, and then detected by incubation for 4 h at RT in a solution containing 0.8 nm colloidal gold-labelled anti-rabbit IgG antibody (Biovalley, Marne la Vallée, France) diluted 1:100 in PBS-1%BSA. For intensification of the gold particles by silver, a commercial kit HQ Silver™ Enhancement (Nanoprobes, NY, USA) was used according to the recommendations of the manufacturer. At the end of immunodetection procedure, half of the sections were observed under a light microscope Zeiss Axiovert 200 M microscope (Göttingen, Germany) equipped with a black and white AxioCam MRm camera, while the other half of the labelled sections were washed in 0.1 M sodium cacodylate buffer, post-fixed in 2% OsO₄ / 0.1 M sodium cacodylate buffer (v/v) for 1 h at RT, dehydrated through an alcohol ascending series and embedded in epoxy resin (Epoxy-Embedding Kit, Sigma Aldrich) polymerized at 60°C during 48 h. Medial preoptic areas were selected and sectioned using an ultramicrotome (Leica Ultracut). Ultrathin sections (70 nm) were contrasted with uranyl salts, and then observed under a transmission electron microscope (80–120 kV EM 912 Omega ZEISS) equipped with a digital camera (Veleta Olympus).

Transmission electron microscopy

For ultrastructural observation, mice (n=3 per treatment group) were deeply anaesthetized with a lethal dose of pentobarbital (120 mg/kg, i.p.) and were transcardially perfused with a 0.9% saline solution following by a 2.5% glutaraldehyde solution diluted in 0.1 M cacodylate buffer pH 7.4. Brains were removed and post-fixed in the same fixative solution overnight at 4°C, then in 2% OsO₄ / 0.1 M cacodylate buffer (v/v) for 1 h at RT. After washes in cacodylate buffer, four coronal sections included mPOA (100-µm thickness) were processed for embedding in epoxy resin as described above.

Fluorescent immunohistochemistry

The animals (n=5 per treatment group and per immuno-labelling) were deeply anaesthetized with a lethal dose of pentobarbital (120 mg/kg, i.p.) and then transcardially perfused with 0.9% saline solution followed by 4% paraformaldehyde solution diluted in 0.1 M PB pH7.4. Brains were removed, post-fixated with the same fixative solution overnight at 4°C, and then cryoprotected

with a 20% sucrose solution for 24 h at 4°C before freezing in isopentane (-30°C). Six to eight serial frozen sections (20 µm thick) including the mPOA were cut using a cryostat and collected on slides. For immune-labelling procedure, non-specific sites were blocked by incubating slide-mounted sections in PBS 1X, 1% BSA and 0.2% Triton X-100 for 1 h at RT. Then sections were incubated with primary antibodies (Table 1) overnight at 4° C diluted in the same phosphate buffer saline (PBS)/bovine serum albumin (BSA)/Triton X-100 solution. Immune complexes were revealed using secondary Alexa-conjugated anti-rabbit IgG (1:1000; Invitrogen, Villebon sur Yvette, France). Fluorescence was observed with a confocal microscope.

Confocal microscopy

Simple and multiple fluorescent labelling was visualized with a SP5 upright Leica confocal laser scanning microscope (Leica Microsystems) equipped with the Acousto-Optical Beam Splitter (AOBS) and using 63x oil immersion objective. Alexa 488 was excited at 488nm and observed from 495 to 580 nm; Alexa 555 was excited at 555 nm and observed from 599 to 680 nm. The gain and offset for each photomultiplier were adjusted to optimize detection events. Images (1024x1024 pixels, 16 bits) were acquired sequentially between stacks to eliminate cross-over fluorescence. The frequency was set up at 400 Hz and the pinhole was set at 1 Airy. Each optical section (1 µm) was frame-averaged four times to enhance the signal/noise ratio. Overlays, projection of the z-stack files and quantification were performed using the Fiji software (NIH, USA). The presented pictures were the projection of 10–20 successive optical sections into one image, unless otherwise stated in the figure legend. Quantification of the fluorescent density was performed on three sections sampled at the level of the mPOA (plate 30 of the Mouse Brain Atlas of Paxinos and Franklin 2001). The surfaces on which the fluorescence density quantifications were performed are given in the figure legends.

Western blot protein analysis using cerebral capillary-enriched fractions

Cerebral capillary-enriched fraction procedure

Brains from 12 mice per treatment group were freshly removed, and two hypothalami were pooled and placed quickly on ice (n = 6 per treatment group). Hypothalamic microvessels were

harvested according to the previously described method (Atallah et al., 2017; Sandoval and Witt, 2011). Briefly, samples were homogenized in a pH 7.4 buffer containing 1% BSA, 2.7 mM KCl, 137 mM NaCl, 1.5 mM KH_2PO_4 , 8 mM Na_2HPO_4 , 1 mM CaCl_2 , 0.5 mM MgCl_2 , 6H₂O, mM D-glucose, 1 mM sodium pyruvate, 1 M 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES). Homogenates were centrifuged in an equal volume of 30% Ficoll for 15 min at 4800g at 4 °C, supernatants were aspirated and pellets suspended in isolation buffer without BSA and passed through a 70- μm nylon filter. Filtrates were centrifuged for 10 min at 4 °C at 3000g.

Protein extraction

Protein extraction from pellets of capillary-enriched fractions was performed with a RIPA buffer containing 50mM Tris—base (pH 7.2), 10 mM EDTA, 10 mM EGTA, 150 mM NaCl, 0.1% sodium dodecyl sulfate, 0.5% deoxycholate acid, 1% Triton X-100, and 1% protease inhibitor cocktail (Sigma Aldrich) and sonicated 10 times for 30 s. Homogenate samples were centrifuged at 13000 rpm for 13 min at 4 °C and supernatants containing proteins were collected. The total protein concentration of each sample was determined using the Bradford Assay Kit (Thermo Scientific, Courtaboeuf-Villebon sur Yvette, France) according to the manufacturer's protocol. Protein extracts were stored at -20°C until further processing.

Electrophoresis and immunoblotting

Protein samples were denatured in Laemmli Buffer and heated at 95°C for 5 min. Electrophoretic migration of 10–20 μg of proteins was carried out on NuPAGE 4–12% Bis–Tris Gel (Invitrogen, Villebon sur Yvette, France). The resolved proteins were then electrotransferred onto pre-treated polyvinylidene difluoride (PVDF) membranes (Millipore, Molsheim, France).

Membranes with transferred proteins were blocked for 1 h, at RT with a solution of 5% non-fat milk diluted in PBS 1X with 0.2 % Tween, and then incubated with primary antibodies (Table 1) diluted in the same blocking solution overnight at 4 °C. Primary antibody binding to blots was detected by incubation with respectively either secondary HRP–conjugated (1:5000; Jackson, Cambridgeshire, United Kingdom) or biotin–conjugated (1:2000; Vector, Burlingame, United States,) anti-rabbit or anti-mouse for 2 h, at RT, and then immune complexes were revealed by

the SuperSignal™ West Pico or Femto Chemiluminescent Substrate kit (Thermo Scientific, Courtaboeuf-Villebon sur Yvette, France).

The signals were quantified by using Fiji software (NIH, USA) and normalized to the value obtained for the corresponding housekeeper glyceraldehyde-3-phosphate dehydrogenase (GAPDH) protein band.

In situ Zymography

In situ zymography was used to check the gelatinase proteolytic activity of MMP-2 and MMP-9 in brain tissue by using a commercial kit (EnzChek® Gelatinase/Collagenase Assay Kit, Thermo Fisher Scientific, Illkirch, France) following the manufacturer's instructions as previously described (Shu et al., 2015). Briefly, after a deep anaesthesia in mice (n= 5 per treatment group) with a lethal dose of pentobarbital (120 mg/kg, i.p.), brains were freshly removed and immediately frozen in isopentane (−30°C). Six to eight serial frozen sections (20-µm thickness) including the mPOA were cut and collected on slides. Four sections for each brain were processed for gelatinase activity and one section for each brain were processed for testing the specificity of the reaction using the metalloproteinase inhibitor, 1,10-phenanthroline, supplied in the kit. For gelatinase activity, cryosections were incubated in a humidity chamber for 1h30 at 37°C in the reaction buffer which contained 30 µg/ml of FITC-labeled DQ-gelatin. For gelatinase inhibitor assays, cryosections were incubated in a humidity chamber for 1 h 30 at 37°C in a reaction buffer which contained 0.5 mM 1,10-phenanthroline with the final substrate concentration of 30 µg/mL DQ-gelatin. Then, sections were rinsed and mounted with mounting medium, then imaged using a Hamamatsu NanoZoomer digital slide scanner 2.0 HT (Hamamatsu Photonics, Massy, France) and analysed with the corresponding NDP Viewer software (NDP.view2, Hamamatsu Photonics, Massy, France). The gelatin-FITC is cleaved by gelatinases, yielding peptides which green fluorescence intensity is representative of net proteolytic activity.

Fluoro-Jade® C staining

Fluoro-Jade® C staining of 20 µm-thickness frozen sections including the mPOA was performed according to the manufacturer's instructions (Interchim, Montluçon, France). Briefly, animals

(n=4 per treatment group) were deeply anaesthetized with a lethal dose of pentobarbital (120 mg/kg, i.p.) and slides bearing frozen cut tissue sections were first immersed in a basic alcohol solution consisting of 1% sodium hydroxide in 80% ethanol for 5 min at RT. Sections were rinsed for 2 min in 70% ethanol, for 2 min in distilled water, and then incubated in 0.06% potassium permanganate solution for 10 min. Following a 1-2 min water rinse, sections were then transferred for 10 min to a 0.0004% solution containing Fluoro-Jade® C dissolved in 0.1% acetic acid. Sections were then rinsed through three changes of distilled water for 1 min per change, dried and then cleared in xylene for at least 1 min. Fluorescence was analysed by using a Hamamatsu slide scanner 2.0 HT, followed by analysis with the NDP Viewer software.

Statistical analysis

The four sample sizes corresponding to the vehicle-treated, DEHP-5 or DEHP-50 and phthalate mixture were equal. Normal distribution of the four groups was checked using the Shapiro-Wilk normality test then one-way ANOVA was used to analyse the main effects of exposure and Tukey tests were used for posthoc analyses to determine group differences. Differences were considered statistically significant if $p \leq 0.05$. In the text, means \pm S.E.M are expressed as a percentage of the vehicle group.

Results

Effect of DEHP alone or in an environmental mixture on AR and ER α in cerebral capillaries of the mPOA

We first looked for the presence of AR and/or ER α in capillaries located in the mPOA. Immunodetection using colloidal gold particles intensified by silver performed on brain sections from untreated mice, showed the presence of AR and ER α in parenchymal cell nuclei in the hypothalamic mPOA, but also exhibited the presence of those sex steroid hormone receptors in capillary walls (Fig. 1 A and B). Ultrastructural observation revealed their presence, at least partly, within cerebral endothelial cells, free in cytoplasm or closely associated with plasma membrane or organelles such as mitochondria (Fig. 1C-F).

Then, we investigated whether AR and/or ER α protein amounts were affected in hypothalamic brain vessels of male mice exposed to DEHP alone or in a phthalate mixture compared to vehicle-treated group.

Data from Western blot performed on microvessel-enriched fractions obtained from the whole hypothalamus (Fig. 2), highlighted a treatment effect on AR protein amount ($p \leq 0.05$), with less AR for both DEHP-50- and Mix-treated mice (-50 %, $p \leq 0.05$) versus the vehicle group following posthoc analyses (Fig. 2A). No significant effect of treatment was found on the protein amount of ER α in hypothalamic capillaries of phthalate-treated groups compared to vehicle (Fig. 2B).

Effects of DEHP alone or in an environmental mixture on metalloproteinases MMP-2 and MMP-9 activity and protein levels

MMPs such as gelatinases MMP-2 and MMP-9, are calcium-dependent zinc-containing endopeptidases. The proteolytic properties of MMPs determine the extent of ECM protein degradation and tissue remodeling surrounding cerebral capillaries. Here, we asked whether exposition to DEHP alone or in a mixture could lead to a dysregulation of MMP-2 and/or MMP-9 activity and/or affect their amounts.

In situ gelatinase zymography study revealed higher level of gelatinolytic activity in the mPOA after phthalate treatment compared to vehicle ($p \leq 0.01$, Fig. 3A-D). Results showed a significant increase of the number of gelatinase-positive cells with more labelled cells for DEHP-5- (+ 34 %, $p \leq 0.05$), DEHP-50- (+ 48 %, $p \leq 0.01$) and Mix-treated mice (+ 38 %, $p \leq 0.01$) versus the vehicle group following posthoc analyses (Fig. 3E). To ensure the specificity of the signal, some representative sections from the four groups were treated with the MMP inhibitor, 1,10-phenanthroline, known to reversibly chelate zinc ions which are essential for MMP activity. Phenanthroline-treated sections showed an absence of signal (Fig. 3A-D, inserts). Western blot analysis performed on hypothalamic microvessel-enriched fractions, showed no treatment effect on the protein amounts of both MMP-2 and MMP-9 (Fig. 3F and G, respectively).

Effects of DEHP alone or in an environmental mixture on the vascular basement membrane integrity

The basement membrane constitutes a continuous network of extracellular matrix included, in particular, the type IV-collagen and laminin. The integrity of the perivascular basement membrane is essential for the BBB integrity. We investigated the distribution and amount of these major components, laminin α -1 and type IV-collagen α -1 (COL-IV α -1), assessed using immunofluorescence and Western blot analysis on hypothalamic microvessel-enriched fractions. Fluorescent analysis revealed an effect of treatment ($p \leq 0.001$, Fig. 4A) on laminin α -1 immunoreactivity surrounding capillary walls and posthoc analyses showed a significant high decrease for DEHP-5-, DEHP-50- and mixture-treated mice in the mPOA (DEHP-5 group: -68 % $p \leq 0.0001$, DEHP-50-group: -60 % $p \leq 0.001$ and mixture group: -69 % $p \leq 0.0001$ vs. vehicle, Fig. 4B). These results were confirmed by Western blot analysis performed on microvessel-enriched fractions showing a treatment effect in hypothalamic vessels ($p \leq 0.01$), with a decrease by -50 % ($p \leq 0.05$) in DEHP-5- and DEHP-50- group, and -61 % ($p \leq 0.01$) in mixture-group, determined by posthoc analyses (Fig. 4C).

Western blot analysis revealed also an effect of treatment ($p \leq 0.001$) on COL-IV α -1 amount with a significant decrease in hypothalamus microvessel-enriched fraction of DEHP-5-, DEHP-50- and mixture-treated groups (-50 % $p \leq 0.05$ vs. vehicle) determined by posthoc analyses (Fig. 4D).

Ultrastructural observation revealed a clean homogenous, compact and electron-dense perivascular basement membrane surrounding endothelial cells for the vehicle group (Fig. 5A), whereas it appeared fainter and sparser for DEHP-5-, DEHP-50- and mixture-treated mice, and associated with an astrocyte end-feet swelling in contact with this basement membrane (Fig. 5B-D).

Effects of DEHP alone or in an environmental mixture on extracellular matrix receptors

Dystroglycan and integrin represent the main receptors / adhesion proteins which are involved in the cell-matrix interactions, thereby support BBB integrity. In order to determine if phthalates are involved in the impairment of cell-matrix interaction, we have investigated the distribution of β -

dystroglycan using immunofluorescence and protein amounts of β -dystroglycan, α -dystroglycan and β -integrin using Western blot analysis on hypothalamus microvessel-enriched fractions.

A significant decrease of β -dystroglycan immunofluorescence density for DEHP-50-treated mice was observed in the mPOA area of brain sections (Fig. 6A), and determined by posthoc analyses (- 60%, $p < 0.05$ vs. vehicle, Fig. 6B).

No significant difference in the β -dystroglycan, α -dystroglycan and β -integrin protein amounts were measured in the hypothalamus microvessel-enriched fraction of phthalate-treated mice compared to those of vehicle-treated mice (Figure 6C-E).

Effects of DEHP alone or in an environmental mixture in neurodegenerative process

Both dysfunction of the BBB and neuroinflammatory status affect brain health, thus providing a starting point of neurodegenerative process (Noe et al., 2020; Palmer, 2011). To assess putative cellular damage induced by phthalate-generated BBB impairment, we used the Fluoro-Jade® C fluorescent marker which is commonly described as a useful specific tool to identify degenerating neurons, activated astrocytes, and microglia during a chronic neuronal degenerating process (Damjanac et al., 2007).

The results showed an effect of treatment with a significant increase of the number of Fluoro-Jade® C -labelled cells ($p \leq 0.01$), highlighting more labelled cells for DEHP-5 (+84 %, $p \leq 0.05$), DEHP-50 (+89 %, $p \leq 0.01$) and Mix-treated mice (+74 %, $p \leq 0.05$) versus the vehicle group following posthoc analyses (Fig. 7A and B).

Electron microscopy study revealed the presence of degenerated cells for DEHP-5-, DEHP-50- and mixture-treated mice in the mPOA, which were morphologically identified as damaged neurons (Fig. 7C).

Discussion

Our previous study demonstrated that DEHP exposure alone or in environmental mixture is associated with an impairment of the NVU in testosterone-sensitive brain regions including the hypothalamic mPOA (Ahmadpour et al., 2021). The present study aims to explore mechanisms involved in these phthalate-induced alterations.

Anti-androgenic action of low doses of phthalates during adulthood exposure has been previously characterized in male mice by a downregulation of neural AR, but not ER α , in the hypothalamic median preoptic nucleus of male mice leading to a sexual behavior disruption (Dombret et al., 2017). ARs and ERs are commonly detected in nuclei of neural cells, neurons and astrocytes (Lorenz et al., 2005) especially in the hypothalamic mPOA in male (DonCarlos et al., 2006). Our present data show, for the first time to our knowledge, the presence of cytoplasmic AR and ER α receptors in capillary wall of adult male mice, suggesting they could also be targets for anti-androgenic endocrine disruptors. We show that exposure to DEHP at 50 $\mu\text{g} / \text{kg} / \text{day}$ or at 5 $\mu\text{g} / \text{kg} / \text{day}$ in a phthalate mixture affects the expression of the AR but not ER α protein in the capillaries of the hypothalamus in male mice. ARs and ERs respective signaling pathway modulate several CNS activities not only by a genomic action but also by a non-genomic one (Patchev et al., 2004). Thus, our results highlight a possible involvement of the AR non-genomic signaling pathway which could lead to a rapid vascular response to androgen-signaling disruptors compounds in this testosterone-sensitive brain area.

AR non-genomic activity may ultimately serve to influence AR genomic activity (Bennett et al., 2010). AR interacts with a large number of co-regulators which function as transactivation chaperones (see for review, Bennett et al., 2010). For instance, among these AR co-regulators, caveolin-1 isoform protein (Cav-1), an integral caveolae structural protein present in endothelial cells and astrocytes, is a well-known AR's co-activator through its transient interaction with Cav-1 (Ikezu et al., 1998). This interaction may be necessary prior to non-genomic AR activity originating from caveolae structures (Lu et al., 2001). We have previously shown that an adult exposure to low doses of DEHP alone or in an environmental phthalate mixture, triggers a significant high decrease of Cav-1 in the hypothalamus (Ahmadpour et al., 2021). Phthalates-induced downregulation of AR and/or Cav-1 in brain capillaries could lead to less interaction between Cav-1 and AR and so to a lower AR transactivation.

Besides that, Cav-1 protein inhibits nitric oxide (NO)-mediated MMPs activity and Cav-1 protein deficiency increases gelatinase activity such as matrix metalloproteinases 2 and 9 (MMP-2, MMP-9) (Gu et al., 2012). Our present results show a significant increase of the MMP-2 and MMP-9

gelatinase activity in the hypothalamic mPOA following exposure to DEHP at 5 µg / kg / day alone or in a phthalate mixture and to DEHP at 50 µg / kg / day. In addition, similar phthalate exposure induces an increase of inducible nitric oxide synthase (iNOS) protein in the (Ahmadpour et al., 2021), suggesting an increase of NO production. Thus, the present observed increase of the activity of MMPs could be due, at least partly, to the decrease in Cav-1 protein levels leading to a disinhibition of NOS and an increase of the production of NO. Our results are in accordance with those suggesting that the Cav-1 / nitric oxide / MMP pathway could be considered as a therapeutic target to ensure vascular protection (Chen et al., 2018).

Furthermore, gelatinases MMP-2 and MMP-9 have been shown mediating the rearrangement and degradation of several endothelial tight junction proteins including ZO-1 (Bauer et al., 2010; Higashida et al., 2011). In our previous work, we have also demonstrated that the increased BBB permeability coincides with the reduction of the tight junction accessory protein ZO-1 level in hypothalamus microvessels following exposure to DEHP at 5 µg / kg / day alone or in a phthalate mixture and to DEHP at 50 µg / kg / day (Ahmadpour et al., 2021). This increased gelatinolytic activity that we report in this present study, could mediate the rearrangement and/or degradation of ZO-1, leading subsequently to the BBB leakage (Bauer et al., 2010; Higashida et al., 2011; Qiu et al., 2011).

Metalloproteases such as gelatinases have been shown to contribute to BBB leakage and neuroinflammation by mediating the degradation of the extracellular matrix surrounding cerebral blood vessels (Rempe et al., 2016; Thomsen et al., 2017). Basement membrane proteins laminin and collagen IV, synthesized predominantly by brain microvascular endothelial cells, pericytes and astrocytes, play a pivotal role in vascular integrity including maintain of the BBB (Chen et al., 2013; Jeanne et al., 2015). In the mPOA, exposure to DEHP alone or in an environmental phthalate mixture induces a decreased immunoreactivity for laminin α -1 and decreased protein levels of both laminin α -1 and collagen IV based on analysis of Western blot performed on hypothalamic microvessel-enriched fractions. Thus, the increase of the gelatinase activity following phthalates exposure may lead, in this hypothalamic brain area, to an alteration / remodeling of the ECM

surrounding brain capillaries which could be also responsible of the neurovascular unit impairment.

The BBB integrity is mainly ensured by cell-basement membrane interactions involving, at least partly, the cellular adhesion receptors such as the β -integrin protein and the dystroglycan complex (Baeten and Akassoglou, 2011). In the brain, different neuronal subtypes and glial cells express dystroglycan (Zaccaria et al., 2001) and integrin is expressed by all the components of the NVU (Baeten and Akassoglou, 2011). The β -integrin receptor mediates anchoring of brain endothelial cells to extracellular matrix components including laminin and Col-IV (Edwards and Bix, 2018; Engelhardt, 2011) but is not affected by phthalates exposure. The dystroglycan complex is constituted by two subunits α and β formed by proteolytic cleavage of a common precursor, and is expressed in perivascular astrocytes, neurons, and endothelial cells. The subunit α -dystroglycan is an extracellular subunit binding to extracellular matrix components such as laminin, whereas β -dystroglycan is a transmembrane protein that anchors α -dystroglycan and actin cytoskeleton, as well as proteins involved in the formation of membrane microdomains such as caveolae (Montanaro and Carbonetto, 2003; Sharma et al., 2010). Our present data show that the alteration of the basement membrane in the mPOA is concomitant to a decrease of β -dystroglycan following exposure to DEHP at 50 $\mu\text{g}/\text{kg}/\text{day}$. The glio-vascular connection which is mediated by the basement membrane, have a crucial role in the integrity, formation and maintenance of the blood-brain barrier (Ezan et al., 2012; Wolburg et al., 2009). In our previous study (Ahmadpour et al., 2021), we have observed a decrease of the astrocytic GFAP in the hypothalamic capillary-enriched fractions which could correspond to a decrease into astrocyte end-feet, a clue of astrocyte-capillaries decoupling. It may originate from the basement membrane impairment and basal membrane receptors alteration caused by MMP-2 and MMP-9 activation. Dystroglycan can be altered by MMPs, thus amplifying several pathogenic states (Hayward and Gordon, 2018) and causing BBB dysfunction (Zhang et al., 2019). β -dystroglycan and tight junction-associated ZO-1 protein interacting together through the actin cytoskeleton. In addition, dystroglycan participates in the astrocyte-mediated fluid homeostasis through

involvement in polarization and distribution of the water-channel protein aquaporin-4 in perivascular astrocytic end-feet (Noell et al., 2011).

Astrocytes also support the neurons in the vicinity of the endothelial cells by regulating of blood flow, fluid, ion, pH, neurotransmitter homeostasis and, energy and metabolism regulation (Sofroniew and Vinters, 2010). Given their strategic location and their function, simultaneous communication with neurons and microvessels, astrocyte morphological and/or function changes may precede or be accompanied by neuronal damage (Zlokovic, 2008). Neurodegeneration in the mPOA, was higher in phthalate-treated mice compared to vehicle. Thus, astrocyte activation, leading to inflammatory mediator production, and MMP activation, leading to the neurovascular unit impairment, could be responsible to the present observed neuronal degenerative process in the mPOA (Rempe et al., 2016), as inflammatory mediators can serve as signaling molecules to induce neurodegeneration (Noe et al., 2020).

A decrease of interaction between AR and Cav-1 protein in brain capillaries, a consequence of their level decrease induced by phthalates, leads to the activation of gelatinases which, in turn, generate alteration of the basement membrane and of cell-matrix interactions (Table 2), resulting in a disruption of the neurovascular unit and neuronal damages. This lesser AR / Cav-1 interaction would also lead to an excessive amount of NO produced following phthalate-induced disinhibition of iNOS in astrocyte end-feet associated with capillaries (Ahmadpour et al., 2021). NO is a signalling free radical involved, among others, in most pathologic features associated with a neurodegenerative and neuroinflammatory status (Saha and Pahan, 2006).

This proposed androgen receptor-mediated cellular pathway (Figure 8), would lead to a failure of the glio-neurovascular coupling which could be involved in the phthalate-induced impairment of the male sexual behavior. These data also raise the question of the involvement of anti-androgenic endocrine disruptors in the onset and/or progression of neurodegenerative processes and complications of many brain diseases and injuries (Baeten and Akassoglou, 2011; Liebner et al., 2018; Sweeney et al., 2018).

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Figure 1

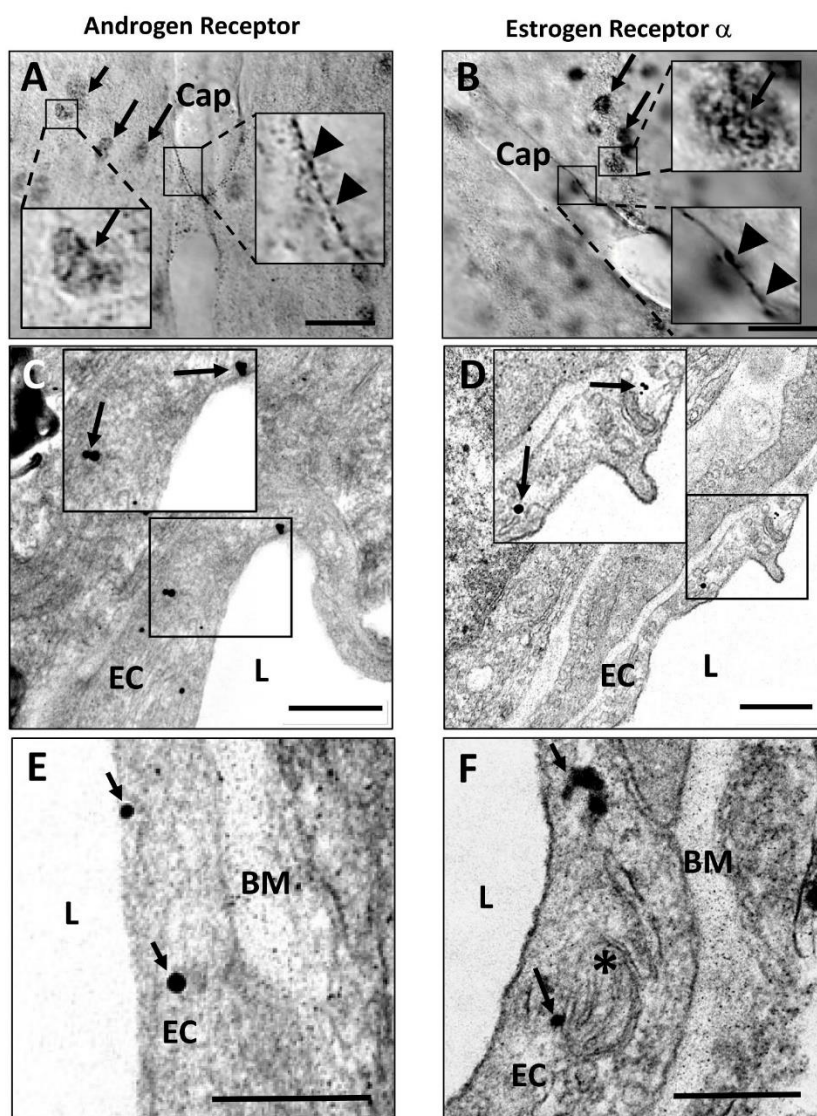


Figure 2

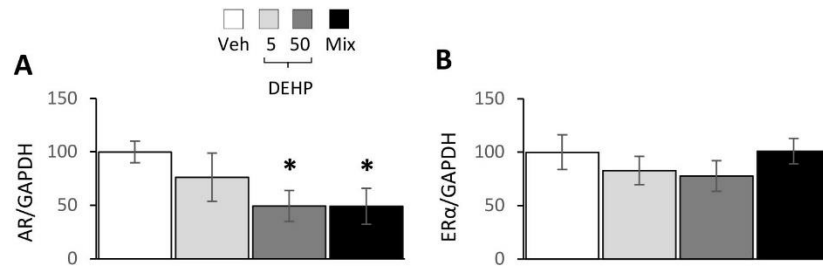


Figure 3

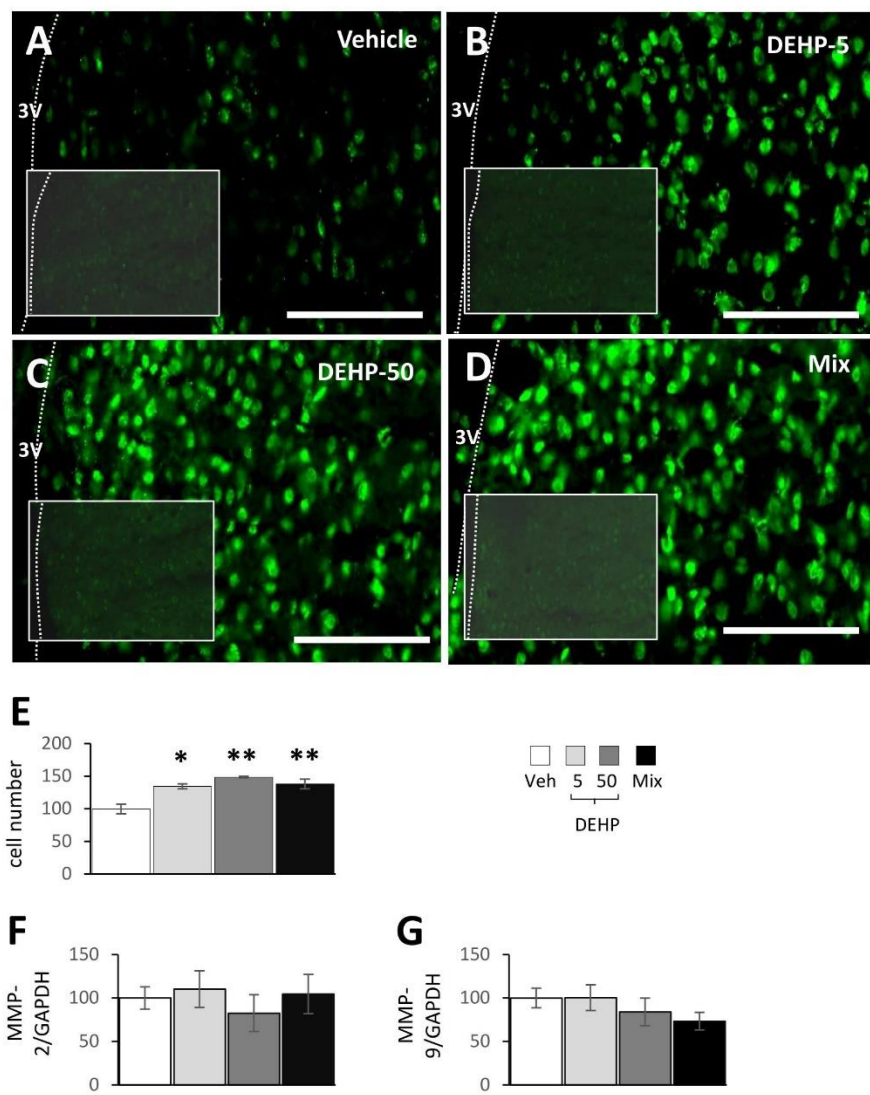


Figure 4

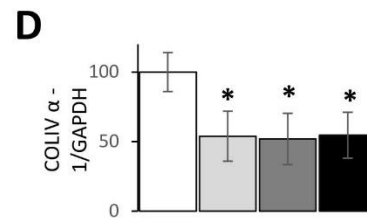
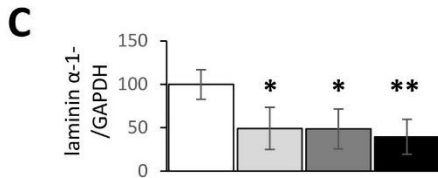
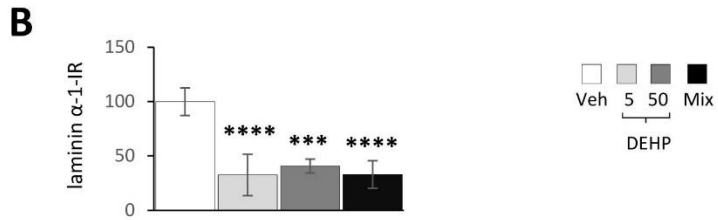
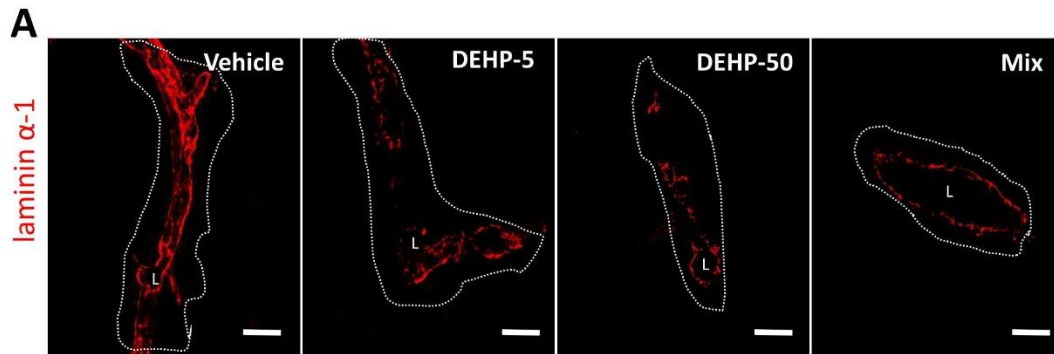


Figure 5

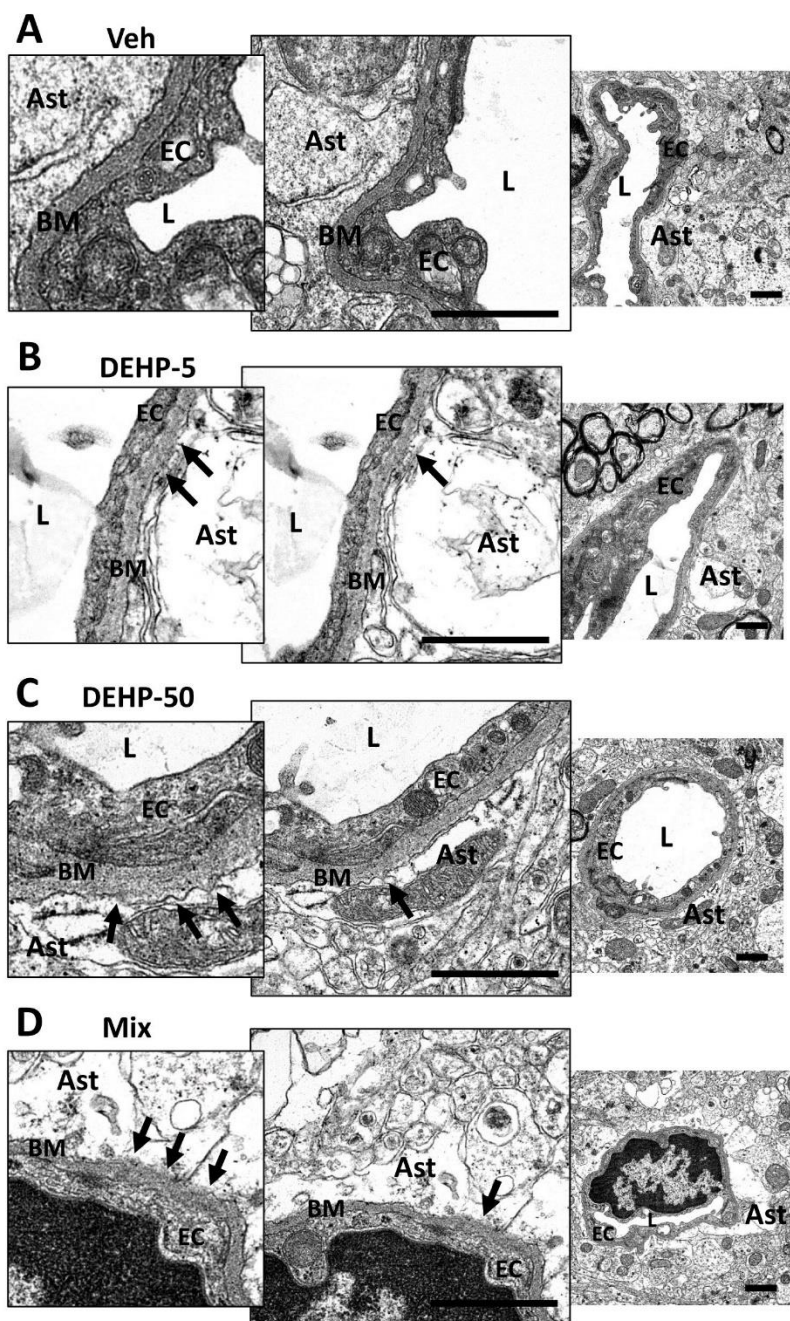


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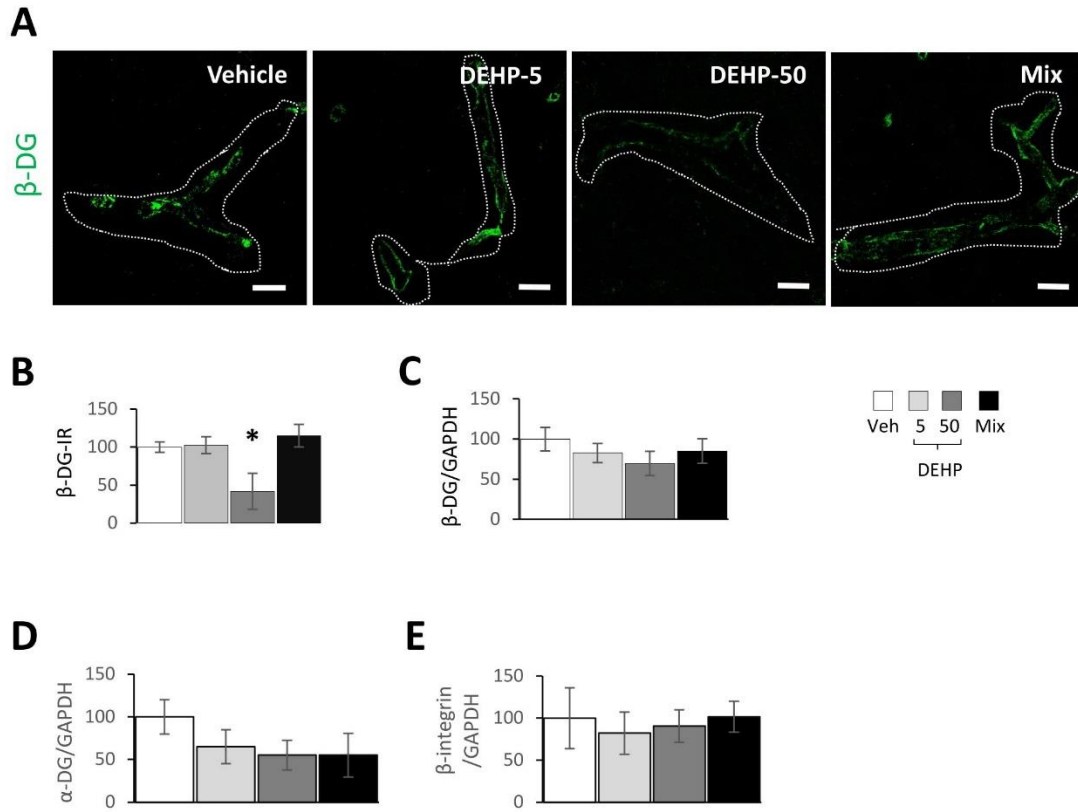
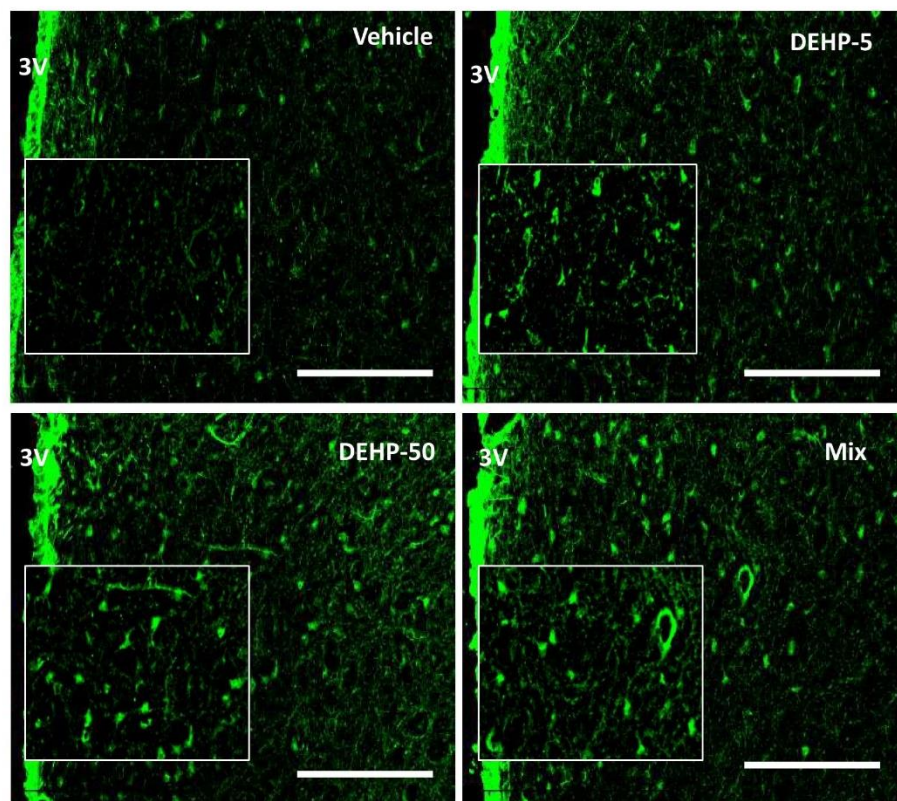
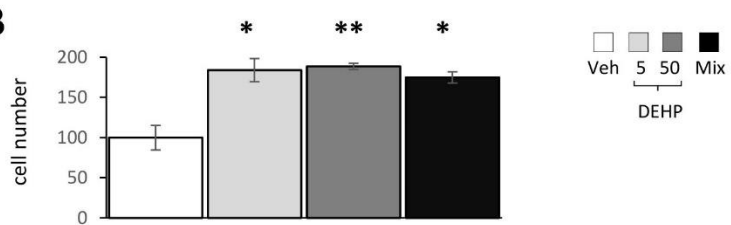


Figure 7

A



B



C

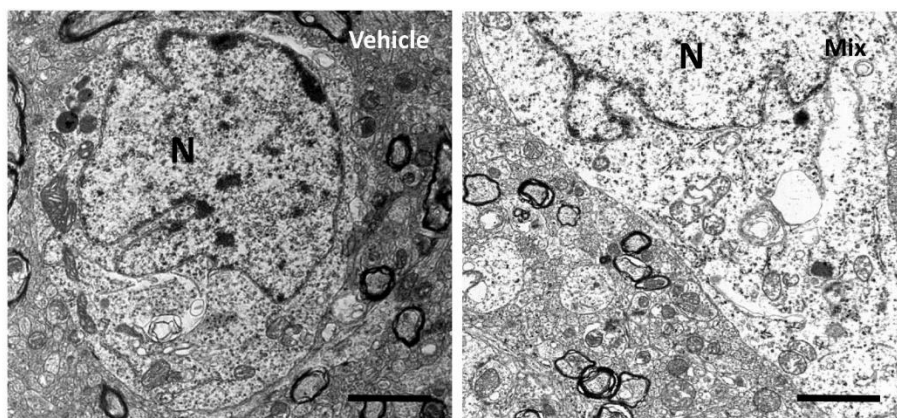


Figure 8

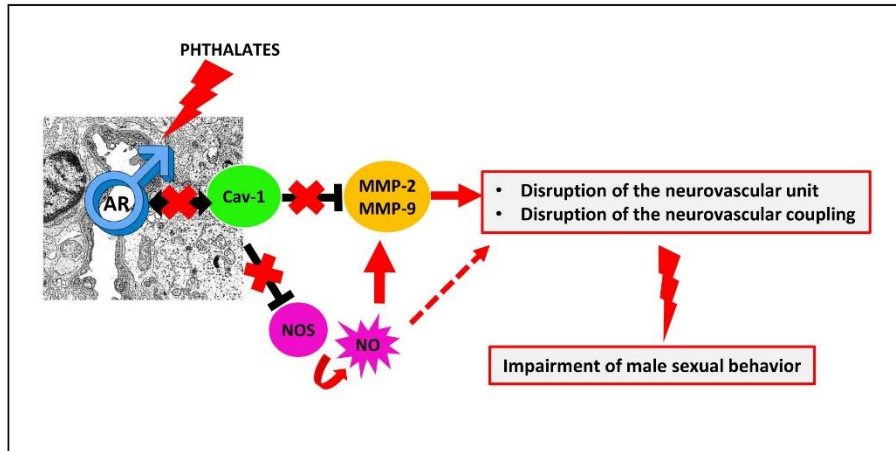


Figure 1. Detection of sex steroid hormone receptors AR and ER α in the mPOA in capillaries of adult male mice

Colloidal gold particles intensified by silver exhibit AR (A) and ER α (B). Arrows: Nuclear receptors, arrows head: receptors in the blood vessel wall. Scale bar: Cap: blood capillary. Scale bar: 10 μ m. Ultrastructural observation using transmission electron microscopy show that both AR and ER α receptors are present in the cytoplasm of endothelial cells of capillaries in the mPOA. The presence of AR (C, D, arrows) is observed, at least partly, within cerebral endothelial cells, free in cytoplasm or closely associated with the luminal plasma membrane. The cytoplasmic ER α (D, F, arrows) is detected in the cytoplasm, or associated to the outer membrane of the mitochondria. Scale bar: 500 nm. BM: Basement membrane; EC: Endothelial cell; L: Lumen

Figure 2. Effects of DEHP alone or in a phthalate mixture on AR and ER α protein levels in cerebral capillaries of the hypothalamus of male mice

Western blot analysis (n = 6 per treatment group) of AR (A) and ER α (B) performed on microvessel-enriched fractions from hypothalamus of mice exposed to the Vehicle (Veh), DEHP at 5 μ g/kg/d (DEHP-5), DEHP at 50 μ g/kg/d (DEHP-50) or the phthalate mixture (Mix). All data are expressed as mean percentages \pm S.E.M of vehicle (100%). * p < 0.05 compared to the vehicle group. Data were normalized to GAPDH level.

Figure 3. Effects of DEHP alone or in a phthalate mixture on MMP-2 and MMP-9 activity and their corresponding protein levels

Representative images of *in situ* zymography (n = 5 per treatment group, green fluorescence) without MMP inhibitor, in the mPOA of vehicle-treated (A), phthalates-treated mice by DEHP at 5 μ g/kg/d (DEHP-5; B), DEHP at 50 μ g/kg/d (DEHP-50; C) and a phthalates mixture (Mix; D) or with the MMP inhibitor 1,10-phenanthroline, (A-D, inserts). Scale bar = 100 μ m

The corresponding quantitative analysis of the gelatinase activity-labelling positive cell number (E) and protein amount of MMP2 (F) or MMP9 (G) based on Western blotting performed on microvessel-enriched fractions from hypothalamus (n = 6 per treatment group). Data of Western blotting were normalized to GAPDH level.

All data are expressed as mean percentages \pm S.E.M of vehicle (100%). * p < 0.05; ** p < 0.01; compared to the vehicle group.

Figure 4. Effects of DEHP alone or in a phthalate mixture on proteins of the basement membrane surrounding endothelial cells of cerebral capillaries in the mPOA of male mice

Representative images (A) and corresponding quantitative analysis (B) of the six to eight serial sections for each brain examined of the immunolabeling of laminin α -1 (red) in the mPOA of mice exposed to the vehicle (Veh), DEHP at 5 μ g/kg/d (DEHP-5), DEHP at 50 μ g/kg/d (DEHP-50), or phthalate mixture (Mix) (n = 5 per treatment group). Scale bar: 10 μ m. L: Lumen. The quantifications of fluorescence density were measured over the surface delimited by the dotted lines.

Western blot analysis (n = 6 per treatment group) of laminin α -1 (C) and COLIV α -1 (D) performed on microvessel-enriched fractions from hypothalamus. Data were normalized to GAPDH level.

All values represent mean percentages \pm S.E.M of vehicle (100%). * p < 0.05; ** p < 0.01; *** p < 0.001; **** p < 0.0001 compared to the vehicle group.

Figure 5. Ultrastructural observation of the basement membrane surrounding endothelial cells of cerebral capillaries in the mPOA of male mice following exposure to DEHP alone or in a phthalate mixture

Representative electron micrographs showing capillaries in the mPOA of mice exposed to vehicle (Veh; A), DEHP at 5 μ g/kg/day (DEHP-5; B), DEHP at 50 μ g/kg/day (DEHP-50; C), or phthalate mixture (Mix; D). (A) A clean homogenous, compact and electron-dense perivascular basement membrane is observed surrounding endothelial cells. (B, C, D) A faint, sparse and discontinuous (arrows) perivascular basement membrane is observed surrounding endothelial cells for DEHP-5, DEHP-50- and mixture-treated mice. A swelling of astrocyte end-feet in contact with this basement membrane is also detected.

Scale bar : 1 μ m. Ast: Astrocyte end-foot; BM: Basement membrane; EC: Endothelial cell; L: Lumen

Figure 6. Effects of DEHP alone or in a phthalate mixture on extracellular matrix receptors

Representative images of the six to eight serial sections for each brain examined of the immunolabeling of β -DG (A) (green) and the corresponding quantitative analysis of the β -DG immunoreactivity density (B) of mice exposed to the vehicle (Veh), DEHP at 5 $\mu\text{g/kg/d}$ (DEHP-5), DEHP at 50 $\mu\text{g/kg/d}$ (DEHP-50), or phthalate mixture (Mix) ($n = 5$ per treatment group). Scale bar: 10 μm . The dotted lines delimit the outer contour of the capillaries where the quantifications of fluorescence density were measured. All values represent mean percentages \pm S.E.M of vehicle (100%).

Western blot analysis of β -DG (C), α -DG (D) and β -integrin (E) performed on microvessel-enriched fractions from hypothalamus ($n = 6$ per treatment group). Data were normalized to GAPDH levels. Values represent mean percentages \pm S.E.M of vehicle (100%). * $p < 0.05$; compared to the vehicle group

Figure 7. Effects of DEHP alone or in an environmental mixture in neurodegenerative process in the mPOA of male mice

Representative images of Fluoro-Jade[®] C fluorescent labelling (A) of the four serial sections for each brain examined in the mPOA of mice exposed to the vehicle (Veh), DEHP at 5 $\mu\text{g/kg/d}$ (DEHP-5), DEHP at 50 $\mu\text{g/kg/d}$ (DEHP-50), or phthalate mixture (Mix) ($n = 4$ per treatment group) and corresponding quantitative analysis of the number positive cells for Fluoro-Jade[®] C labelling (B). Scale bar = 100 μm .

(C) Representative electron micrographs showing an intact neuron in the mPOA of mice exposed to vehicle (Veh; left panel), and a damaged neuron in the mPOA of mice exposed to the phthalate mixture (Mix; right panel). Scale bar = 2 μm .

Figure 8. Proposed cellular pathway for glio-neurovascular coupling impairment following adult exposure to low doses of DEHP alone or in an environmental phthalate mixture in male mice

A decrease of interaction between AR and Cav-1 protein in brain capillaries, a consequence of phthalates exposure, leads to the activation of gelatinases which, in turn, generate alteration of the basement membrane and of cell-matrix interactions. This could result in a disruption of the

glio-neurovascular unit and neuronal damages. This lesser AR / Cav-1 interaction would also lead to an excessive amount of NO produced following phthalate-induced disinhibition of iNOS in astrocyte end-feet associated with capillaries. This proposed androgen receptor-mediated cellular pathway could be disrupted in the phthalate-induced impairment of the male sexual behavior.

Table 1: Antibodies used in immunohistochemistry (IHC), transmission electron microcopy immunocytochemistry (TEM-ICC), and Western blot (WB)

Primary Antibody	Host	Company	Catalog N°	Application	Dilution
α -dystroglycan	Rabbit	Abcam	Ab151979	IHC-WB	1/300-1/500
AR	Rabbit	Abcam	Ab133273	WB	1/500
	Rabbit	Santa Cruz Biotechnology, Inc.	sc-816	TEM-ICC	
β -dystroglycan	Rabbit	Santa Cruz Biotechnology, Inc.	sc-28535	IHC - WB	1/200-1/300
ER α	Rabbit	Santa Cruz	sc-542	WB	1/200
	Rabbit	Biotechnology, Inc.		TEM-ICC	
GAPDH	Mouse	Santa Cruz Biotechnology, Inc.	sc-32233	WB	1/10000
β -integrin	Rabbit	Abclonal Technology	A11060	IHC-WB	1/100-1/500
Laminin α -1	Rabbit	Sigma-Aldrich	L9393	IHC - WB	1/200 - 1/100
MMP-2	Mouse	Santa Cruz Biotechnology, Inc.	sc-13594	WB	1/500
MMP-9	Mouse	Santa Cruz Biotechnology, Inc.	393859	WB	1/100

Table 2

Synthesis of the effects of an oral exposure during adulthood to low doses of DEHP alone or in a phthalate mixture in male mice on the neurovascular unit in the hypothalamus mPOA

Sex steroid receptors in capillaries

<i>AR</i>	present; decreased: DEHP-50 and Mix
<i>ER α</i>	present; not affected

Gelatinases MMP-2 and MMP-9

<i>Activity</i>	increased: DEHP-5, DEHP-50 and Mix
<i>Protein level</i>	not affected

Basement membrane components

<i>Laminin</i>	decreased: DEHP-5, DEHP-50 and Mix
<i>Collagen IV</i>	decreased: DEHP-5, DEHP-50 and Mix

Cell / matrix interaction receptors

<i>α-dystroglycan</i>	not affected
<i>β-dystroglycan</i>	decreased: DEHP-50
<i>β-integrin</i>	not affected

Neurodegenerescence	increased: DEHP-5, DEHP-50 and Mix
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6 Discussion

Phthalates are endocrine disruptor chemicals showing effects on endocrine systems including those involved in the control of reproductive functions. They pose a particular high risk to the male reproductive system, due to their interference with testosterone production and/or androgen receptor signaling pathway. Among the phthalate family, the di(2-ethylhexyl) phthalate (DEHP) represents the most abundant compound in the environment. In addition to DEHP, other phthalates including diethyl phthalate (DEP), dibutyl phthalate (DBP), butyl benzyl phthalate (BBP), and diisobutyl phthalate (DiBP) are also detected in the environment worldwide (Gao and Wen, 2016). Recently, our team has reported that chronic low-dose exposure to DEHP during adulthood disrupts the emission of courtship vocalizations and initiation of mating in male mice (Dombret et al., 2017). This alteration is not associated with modification of circulating testosterone levels and/or HPG axis, but with a reduced androgen sensitivity confirmed by the demonstration of androgen receptor down-regulation in the hypothalamic mPOA, the region involved in the expression of the sexual behavior in male.

The male hippocampus also exhibits a high sensitivity to androgens and can thus be targeted by compounds exhibiting anti-androgenic activities. In this context, a previous study has showed that prenatal or perinatal exposure to DEHP at 200 µg/kg/d or 10 to 200 mg/kg/d impaired spatial memory and induced anxiety- and depressive-like behaviour in adult male mice, and that these effects were associated with AR down-regulation in the hippocampus (Barakat et al., 2018; Xu et al., 2015). In parallel, we have shown that reduced androgen sensitivity through testosterone depletion in adult male mice induces glial activation and up-regulation of inflammatory molecules which are associated with an increased blood-brain barrier (BBB) leakage in cerebral capillaries of the mPOA (Atallah et al., 2017).

The BBB is a highly regulated specific interface at the level of cerebral capillaries that maintains cerebral homeostasis to ensure neuronal activity underlying the regulation of all physiological functions. Its dysfunction can lead not only to disruption of neural functions and behaviors but also to complications in many brain diseases, injuries and insults. BBB allows the

control of substance delivery from the blood compartment to the brain parenchyma, as well as the elimination of compounds from the parenchyma to the blood flow. Thus, BBB limits paracellular and transcellular diffusion through endothelial cells (ECs) of capillaries sealed by tight junctions (TJs), and very specific transport systems and low rates of caveolae-mediated transcytosis, respectively. At their abluminal side, ECs are surrounded by basement membrane embedding pericytes, and interact with glial cells (astrocytic end-feet and microglial cells) and neurons. The whole is called the neurovascular unit. The presence of glial cells in the vicinity of the BBB promotes the local susceptibility to neuroinflammatory responses. Neuroinflammation, in turn, contributes to the BBB dysfunction.

Male rat cerebral vessels are target tissues of testosterone as they express androgen and estrogen receptors and enzymes involved in the biosynthesis of estradiol and dihydrotestosterone (Gonzales et al., 2007,2010). Considering that phthalates display an anti-androgenic effect in the brain sensitive regions including mPOA and hippocampus, where testosterone plays a neuroprotective effect, the aim of the present study was to investigate whether phthalates could impact NVU function in these brain sensitive areas. For this purpose, two-month-old C57BL/6J males were exposed orally to DEHP alone (5 or 50µg/kg/d) or to DEHP (5µg/kg/d) in a phthalate mixture. After 6-week exposure, male brains were collected and analyses were performed in order to investigate the integrity of the NVU.

The major results obtained during this thesis show that phthalates impact cerebrovascular physiology in testosterone-sensitive brain areas of adult male mouse (Ahmadpour et al., 2021).

The phthalate-induced disruption of the neurovascular unit may lead to the failure of the glyo-neurovascular coupling which would be supported by the AR / Cav-1 / nitric oxide / MMP pathway. This cellular pathway would involve cerebral capillary-associated AR non- genomic activity.

Based on experimental data, take-home messages can be listed as below:

1- DEHP alone at the TDI dose or at a ten-lower dose, or in combination with other phthalates commonly detected in the environment, triggers the NVU alteration in testosterone sensitive brain regions.

Till now, there is no report available on impact of oral exposure during adulthood to low doses of DEHP alone or in a phthalate mixture and to the best of our knowledge, present studies report for the first time the impairment of NVU integrity following exposure to phthalates.

Exposure to low doses of DEHP alone or in a phthalate mixture increased BBB permeability to exogenous and endogenous tracers and affected the accessory tight junction protein Zonula Occludens-1 and caveolae protein Cav-1 in the mPOA and the hippocampal CA1 and CA3 areas. This was associated with an inflammatory profile including an astrocyte activation and inducible nitric oxide synthase expression in the mPOA, and a microglial activation in the mPOA and the hippocampal CA1 and CA3 areas. The protein levels of the inflammatory molecule COX-2 and iNOS were increased in activated microglial cells and perivascular astrocytic end-feet respectively, in the mPOA.

A dysfunction of the BBB is associated with neurological damage and disease (Zhao et al., 2015) as well as with peripheral diseases such as diabetes (Prasad et al., 2014) and non-neural, inflammatory-related pathologies (Liu et al., 2017b). Alteration of the cells of the NVU leads to BBB dysfunction (Hawkins, 2005) which, in turn, contributes to the progression of neurological diseases (Hawkins, 2005; Reinhold and Rittner, 2017).

As already mentioned, several studies addressed the effects of phthalate exposure on the male's central nervous system, the majority of investigations has focused on prenatal and/or postnatal exposure of DEHP and DBP on neurobehavioral, with reported changes in aromatase activity, neurotransmitter content or expression levels of synaptic plasticity markers (see table 4). However, less is known about the effects of phthalates on the structure and function of the BBB and its close environment. It is important to recognize two significant issues: first, despite this high level of protection, the brain can be reached and exposed to compounds able to cross the

BBB. Second, as observed, the BBB and its cellular and non-cellular constituents, can also be targets of environmental toxics, by being subjected themselves to modulation and dysfunction.

2- Similarities and differences exist in the vulnerability of the NVU across regions and / or subregions of the brain

In the hippocampus, in contrast to results obtained for the hypothalamus mPOA, no change in astrocyte activation markers was observed following phthalate exposure. Microglial activation was observed in the CA1 and CA3 sub-regions whereas no change in COX-2 immunoreactivity was demonstrated. Our results have showed that the increased BBB permeability induced by exposure to phthalates was associated with a dramatically decreased amount of the accessory junctional protein ZO-1 in hypothalamic microvessel-enriched fractions. An opposite effect was observed in hippocampal microvessel-enriched fractions in the group exposed to DEHP at 50 µg/kg/day. Further, in the DG, none of the modifications induced by phthalate exposure on BBB permeability and amounts of proteins involved in endothelial TJs and Cav-1 were observed, except for iNOS immunoreactivity. These results suggest that, within the brain, different regions may exhibit differential sensitivity to exposure to DEHP alone or in a mixture.

The differences observed in astrocyte activation and COX-2 expression between the mPOA and the hippocampal CA1 and CA3 regions, and also within the same brain area for the different components of the neurovascular unit could be underlined by different expression patterns of AR and ERs in neurons, endothelial cells, astrocytes and microglia(Hajszan et al., 2007; Kerr et al., 1995; Picot et al., 2016). Indeed, in the male rodent nervous system, testosterone exerts its effects through direct activation of the AR, but can also be metabolized into estradiol, which then stimulates ER α and ER β .

No remarkable change in DG may reflect the less sensitivity of this sub region to sex steroid hormones. The different subregions of hippocampus have different sensibility to testosterone as they as they have different levels of AR expression, with a predominant distribution in CA1 and CA2/CA3 in comparison to DG (Hajszan et al., 2007; Kerr et al., 1995; Raskin et al., 2009). Thus,

the vulnerability of cerebral capillaries to phthalates may be dependent of the AR expression in the considered brain area. Besides, hippocampal subregions possesse distinct neuronal network architectures and afferent pathways which participate in observed hetrogenesity in response to phthalates exposure.

In this context, it is important to stress that the cerebrovascular function in human also seems sensitive to androgen, although these effects are less defined than for estrogens (see for review, (Robison et al., 2019)).

3- Phthalate-induced disruption of the neurovascular unit in testosterone-sensitive brain areas could be involved, at least partly, in alterations of their corresponding androgen-dependant behaviors.

Sexual behavior comprises precopulatory (olfactory preference, emission of ultrasonic vocalizations) and copulatory (mounting, thrusting, intromission, ejaculation) phases, which are tightly regulated by a well-identified neural circuitry. Pheromone cues emitted by a sexually receptive female are detected by the main olfactory epithelium and vomeronasal organ of the male and are transmitted, respectively, to the main and accessory olfactory bulbs, then to chemosensory responsive nuclei in the medial amygdala, the bed nucleus of stria terminalis and finally to the medial preoptic area, where they are processed into behavioral responses. This neural circuitry is highly sensitive to gonadal testosterone, which plays a crucial role in its perinatal organization and adult activation through two main signaling pathways involving AR and ERs (Mhaouty-Kodja, 2018b). This makes this circuitry also sensitive to exposure to exogenous factors exhibiting hormone-mimetic activities such as endocrine disrupters. Indeed, it has been shown circuitry is highly vulnerable to adult exposure to low doses of endocrine disrupters (Capela and Mhaouty-Kodja, 2017; Dombret et al. 2017; Picot et al. 2014). An exposure to DEHP at the tolerable daily intake dose (TDI: 50 µg/kg/day) or a 10-fold lower dose, close to environmental exposure, alters the emission of ultrasonic vocalizations and ability to attract receptive females, and delays the initiation of mating (Dombret et al. 2017). It is important to

indicate that, since 1975, sexual health has been included by the World Health Organization (WHO, 2006) as part of reproductive health, and it is recognized that sexual dysfunction may affect not only reproduction but also mental and general health, well-being, and maturation (Adam et al., 2021).

Cognitive function is the result of high-level neural activity in the human brain. Hippocampus is one of the key parts of brain involved in memory and emotions. The hippocampus receives input from and sends output to the rest of the brain via a structure known as the entorhinal cortex, which is located beneath the anterior (frontal) region of the hippocampus. The hippocampal formation itself is composed of several subregions, which include the cornu ammonis (CA1–4), the dentate gyrus, and the subiculum. The neural circuits of the hippocampus are greatly revised elsewhere (Knierim, 2015). It has been demonstrated that androgen deficiency is closely related to modulation of hippocampal functions and cognitive impairment (Picot et al., 2016). Indeed, the maintaining of memory are greatly related to synaptic plasticity processes within the hippocampal pruned neuronal networks. AR modulate hippocampal synaptic plasticity and healthy memory processes. (Mhaouty-Kodja, 2018b). In addition, in epidemiological studies of healthy older men, higher testosterone concentrations have been associated with better global cognition (Wahjoepramono et al., 2016).

The relationships between the exposure to endocrine disruptor chemicals such as phthalates and cognitive (Schantz and Widholm, 2001) and social behavior deficits (Miodovnik et al., 2011) have been documented. Previous study showed that prenatal or perinatal exposure to DEHP at 200 µg/kg/d or 10 to 200 mg/kg/d impaired spatial memory and induced anxiety- and depressive-like behaviour in adult male mice, and that these effects were associated with AR down-regulation in the hippocampus (Barakat et al., 2018; Xu et al., 2015). Cognitive function is a major determinant of an individual's quality of life. (McDonald, 2017). In addition, with the rapid ageing of the world's population, finding ways to maintain the cognitive vitality of older people has become an increasing concern (Butler et al., 2004).

Exposure to low doses of DEHP alone or in environmental phthalate mixture induces a dysfunction of the neurovascular unit in the mPOA and hippocampus, which could participate, at least in part, to the reported behavioral alteration.

To conclude, endocrine disruptors should be considered as environmental risk factors for cerebrovascular function and these results raise questions about the determination of the TDI for phthalates in humans.

Fertility in humans and wildlife is a major concern of the 21st century. Several studies document a decline in male reproductive health in several locations worldwide (WHO State of the Science of Endocrine Disrupting Chemicals, 2012). This decline, which started in the middle of the 20th century, coincides with a more than 20-fold increase in the use of natural and synthetic chemicals with endocrine disrupting activities. For instance, a relationship between exposure to endocrine disruptors and sperm count and quality in men has been strongly suggested (see for review; Capela and Mhaouty-Kodja, 2017). Furthermore, neurological disorders have become more prevalent in our societies. According to the Global Burden of Diseases, Injuries, and Risk Factors Study in 2016 (GBD, 2016) and other sources of population-level estimate of risk including WHO and UNICEF, brain pathologies have become more prevalent in our societies in the last few decades (GBD 2016 Neurology Collaborators, 2019; Mesnil et al., 2020). Although, a better diagnosis and longer life expectancy partly explain this increase, but emerging data suggest that long-term exposure to pollutants is a possible but still underestimated cause of major brain disorders (Brewer, 2015; Mesnil et al., 2020). For example, the onset of neurodegenerative disease has been shown to be related to exposure to polychlorinated biphenyls, chlorinated pesticides, polybrominated diphenyl ethers as well as to phthalates and bisphenol-A (Zeliger and Lipinski, 2015). Among other, it has been shown that exposure to DEHP leads to serious health concerns. One of these concerns is its possible link to Alzheimer's disease, which is the most common neurodegenerative disease in aging individuals. Available data have shown that early-life and chronic exposure to DEHP contribute to neurodegenerative pathological changes; however, the potential mechanisms remain unclear (Yen et al., 2021).

Our analyses addressing the impact of DEHP exposure on behavioral responses in male (Dombret et al., 2017) and female (Adam et al., 2021) and BBB integrity (Ahmadpour et al., 2021) in male mice show that DEHP-induced effects can be observed at low doses equivalent or below the tolerable daily intake dose of 50 µg/kg/d, which was established and recently updated by the European Food Safety Authority (EFSA) on the basis of reduced fetal testosterone production. This indicates that the nervous system including the neurovascular unit is highly sensitive to phthalates and should be also considered as a relevant endpoint in risk assessment for these molecules.

We and our children are not shielded from the dreadful consequences that can be caused by phthalates exposure and this is a sad observation of the situation. In this context our responsibility is raise awareness and put the accent on the outcome of endocrine disruptor as a public health priority worldwide, to articulate a public health concern and to advocate for action at national and international levels.

It is only through the active and continued pursuit of this challenging research area that we will facilitate policy-makers to address the endocrine disruptors as an increasing threat to global health. As the proverb says: *“Mens sana in corpore sano”*, a healthy mind in a healthy body.

7 Perspectives

The present *in vivo* study shows, for the first time to date, that endocrine disruptors belonging the phthalate family, can interfere with AR mediated-cellular pathways involved in the physiology of cerebral capillaries at doses close to the environmental exposure, and thus suggests that such an oral exposure during adulthood to phthalates may be considered as an environmental risk factor also for the cerebrovascular function.

Exploring the BBB response to chemical endocrine disruptors is critical to get new insights for understanding the link between environmental chemical pollutants and their associated-risk for the glio-neurovascular coupling integrity. Moreover, my results open new aims of research with following questions and perspectives:

- **Does exposure to phthalates induce oxidative stress and modify mitochondrial bioenergetic mechanism at the level of the blood-brain barrier?**

Oxidative stress is a condition that causes the release of superoxidized free radicals (ROS) derived from oxygen such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) etc. When these molecules are synthesized in too large a quantity, they damage proteins, lipids and nucleic acids (DNA) until they are completely degraded (Song et al., 2020). The brain endothelium serves as both a source of, and a target for ROS. Mitochondrial dysfunctions are implicated in neuroinflammation, neurodegenerative mechanisms and neurovascular impairment (Song et al., 2020).

Proteomic and molecular analyzes of the preoptic area of the hypothalamus have revealed that the DEHP-induced AR down-regulation is associated with an alteration of cellular processes such as metabolism, cellular stress and neuroprotection, and mitochondrial function (Dombret et al. 2017). A disruption of mitochondrial energy metabolism could induce a state of oxidative stress. The cerebral capillaries are very rich in mitochondria (compared to the peripheral vascular system), these constitute 8 to 11% of the cytoplasmic endothelial volume of the capillaries (Oldendorf et al., 1977). The large number of mitochondria could be explained by an important

metabolic capacity of brain vessels to maintain cerebral homeostasis. Mitochondria are the main sources of energy production in the cell. The 4 mitochondrial complexes use the electrons supplied by NADH (complex I) or FADH₂ (complex II) in the respiratory chain, the final acceptor of these electrons being O₂ to give H₂O. A proton gradient is formed between the inter-membrane space and the mitochondrial matrix, and is used to operate an ATPase pump to generate ATP. In addition to being the main sources of ROS production, mitochondria are also easy targets of oxidative stress.

Therefore, the BBB constitutes an area very sensitive to the effects of oxidative stress and for the upcoming, it would be quite interesting to investigate the effect of phthalates on oxidative stress and mitochondrial bioenergetics. In this context, investigations have recently been carried out and preliminary results appear to be encouraging (data not shown). The effect of an adult exposure to low doses of DEHP alone or in an environmental phthalate mixture on bioenergetics metabolism has been studied in collaboration with Dr. Aurore L'Honoré (Adaptation Biologique et Vieillesse (B2A), IBPS, Sorbonne Université / INSERM / CNRS). We have adjusted the methodology to use freshly prepared hypothalamus or hippocampus microvessel enriched-fractions from non-exposed and exposed animals using a Seahorse XF24 Metabolic Analyzer. Thus, cellular oxygen consumption rate (OCR), indicating mitochondrial respiration and extracellular acidification rate (ECAR), indicating anaerobic glycolysis, have been analyzed in real time in brain capillary fractions. Moreover, the maximal capacities were measured in the presence of a mitochondrial uncoupler FCCP (carbonyl cyanide 4- (trifluoromethoxy) phenylhydrazone) to stimulate the maximal oxidative phosphorylation rates and an ATP synthase inhibitor oligomycin to activate glycolytic pathway. Proton leak and spare respiratory capacity have been evaluated using rotenone and antimycin A, which are mitochondrial complex I and III inhibitors, respectively. Later, basal and maximal OCR and ECAR as well as total respiratory and glycolytic capacity will be compared.

To complete the analysis of the functioning of the mitochondrial respiratory chain, a real-time polymerase chain reaction (qPCR) has been performed in order to quantify the mitochondrial DNA / genomic DNA ratio (mtDNA / nDNA). The results are being interpreted and

will make it possible to estimate whether exposure to phthalates induces a change in the number of mitochondria in the cerebral capillaries.

As preliminary results tend to show a change in bioenergetic metabolism following exposure to phthalates, we will then evaluate the *in situ* production of ROS using a fluorogenic probe, the superoxide indicator dihydroethidium (DHE), also called hydroethidine, following confocal microscope analysis. DHE functions as a redox-sensitive probe, and is oxidized by superoxide anion to a red fluorescent product (Bindokas et al., 1996).

DNA double-strand breaks and DNA damage induced by reactive oxygen species will be sought by immunohistochemistry using specific antibodies raised to γ H2AX and 8-oxoguanine. In eukaryotes, DNA has a compacted structure. The first stage of compaction of chromatin is the wrapping of DNA around histones such as H2A to form nucleosomes. In the event of a double-stranded DNA break, H2A is called γ H2AX. following phosphorylation on serine 139. 8-Oxoguanine is the most common DNA damage induced by oxidative stress. This lesion modifies guanine and can induce pairing changes between G (guanine) and T (thymine) bases as well as C (cytosine) and A (adenine); it represents an appropriate marker for the evaluation of the effect of ROS on DNA.

These data, with those from several other studies, will complement a larger study conducted in the team on the impact of these endocrine disruptors on oxidative stress.

- **What are the risks and consequences of adult exposure to low doses of phthalates on the neurovascular unit in female?**

The adverse effect of phthalates on women has already been reported. Previous studies in humans reported that environmental exposure to DEHP is associated with low interest in sexual activity in premenopausal women (Barrett et al., 2014). Moreover, it was shown that urinary concentrations of several high-molecular-weight phthalates were associated with later puberty in girls (Berger et al., 2018). Our team has recently revealed that an adult exposure to low doses of DEHP alone or in an environmental phthalate mixture altered female mice sexual behavior which was underlined by a lower number of progesterone receptor (PR)-expressing neurons, without changes in ER α , in both the facilitatory (medial amygdala, bed nucleus of stria terminalis,

ventromedial hypothalamus) and inhibitory (medial preoptic area, arcuate nucleus) systems of lordosis behavior (Adam et al, 2021); up-regulation of PR is required for the induction of female receptivity in rodents (for review: MhaoutyKodja et al. 2018). This down-regulation of neural PR and lowered behavior in females are comparable with results obtained in males (Dombret et al., 2017), and DEHP exposure seems to target a close subfamily of neural steroid receptors, i.e., AR in males and PR in females. Interestingly, AR and PR are closely related receptors in the same steroid receptor subgroup, in addition to mineralocorticoid and glucocorticoid receptors, according to the phylogeny of the steroid receptor gene family (Eick and Thornton, 2011). Whether AR and PR genes share common regulatory mechanisms of expression that could be similarly disrupted by DEHP exposure needs further investigation.

Besides, progesterone is considered to have a neuroprotective effect in ischemic and traumatic brain insult models in female. Previous studies have shown that post-injury treatment with progesterone decreases brain edema, attenuates the damages caused by oxidative stress and neuroinflammatory mediators, and preserves BBB function (Si et al., 2014; Wang et al., 2009). Given the potential protective effects of progesterone in BBB function in females, it is of great interest to investigate the effect of phthalate exposure on the permeability and function of the neurovascular unit in female.

- **What are the risk and consequences of exposure to low doses of phthalates on the developmental and/or neonatal neurovascular unit?**

Several developmental windows are critical in terms of exposure to endocrine disruptors, especially fetal development and early childhood. They are particularly vulnerable to the effects of phthalates, starting with maternal exposure, placental transfer during gestation, at birth in the health-care service as well as following birth and during infancy (Bowman and Choudhury, 2016). Consequently, exposure of phthalates to pregnant women and childhood is highly prevalent. The developing brain and BBB formation is particularly vulnerable to changes in the maternal or fetal environment. BBB development is orchestrated by multiple cellular and molecular mechanisms mediating brain angiogenesis, barrier formation as well as communication between vascular and nervous systems. Impairment of neurovascular development could induce defective function of

blood-brain barrier and alteration of brain homeostasis. Further, the proper function of the BBB during development of the fetus is essential to ensuring optimal brain growth and protection from drugs and toxins once they cross the placenta (Goasdoué et al., 2017). Thus, it would be interesting to investigate the effect of phthalates on the BBB formation, development and function at these critical windows.

- **Are the effects of phthalates on the neurovascular unit permanent or reversible?**

The reversibility of the chronic adult exposure to DEHP has been suggested. Our team has already shown that behavioral alterations and down-regulation of androgen receptor, caused by continuous exposure to low doses of DEHP, could be reversible and restorable by interruption of exposure (Capela and Mhaouty-Kodja, 2017). In this context, it would be interesting to study the reversibility of the adverse effects on the neurovascular unit induced by a subchronic exposure to DEHP alone or in mixture.

Understanding the role of AR signaling in the BBB physiology is, in addition, critical to get new insights in neurological and metabolic diseases involving BBB dysfunction and for the development of drugs that can cross the BBB. Indeed, BBB disruption is a consequence of some acute disorders, such as stroke and traumatic brain injury, and is present in some chronic neurodegenerative diseases like Alzheimer's dementia and Parkinson's disease. In other cases, BBB disruption may be a precipitating event, such as in multiple sclerosis, epilepsy, obesity, or diabetes (see chapter 1). Population-based epidemiological studies showed that high levels of androgens increase the incidence of cerebrovascular disease in young adult men (Wang et al., 2013). The natural decline in physiological levels of circulating androgens also contributes to increased incidence and worsened outcome with aging (Yeap et al., 2009).

8 References

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9 Annex

Review article

[Involvement of Testosterone Signaling in the Integrity of the Neurovascular Unit in the Male: Review of Evidence, Contradictions, and Hypothesis.](#)

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Neuroendocrinology. 2021;111(5):403-420. doi: 10.1159/000509218. Epub 2020 Jun 8.

Involvement of Testosterone Signaling in the Integrity of the Neurovascular Unit in the Male: Review of Evidence, Contradictions, and Hypothesis

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Keywords

Adult male brain · Testosterone · Blood-brain barrier · Neuroinflammation · Neurovascular unit

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 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 was to summarize and discuss the literature, providing insights and contradictions to high-light hypothesis and the need for further investigations.

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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 nonreproductive systems such as the central nervous system (CNS), 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 are 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 (EC) 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 suggests that cerebral blood vessels are directly targeted by sex steroids [5]. The 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 supple-

mentation 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 clarifying 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, to our knowledge no review is available on this subject, and data reveal evidence but also contradictions. Thus, the intention of our review was to synthesize the literature data 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. We then review and discuss data concerning the effects of testosterone on cerebral microvessels, 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 CNS, cells require a stable microenvironment, which is guaranteed by a complex cellular and molecular system involving brain microvessels 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 ECs that are sealed by continuous intercellular molecular binding systems including adherens junctions and tight junctions (TJs) [13] that limit paracellular diffusion [14] (Fig. 1). The major transmembrane components of TJs are claudins, occludin, and junctional adhesion molecules [15]. They interact with the actin cytoskeleton via cytoplasmic TJ accessory proteins, including zonula occludens (ZO) proteins [16] (Fig. 1). In both mice and humans, claudin-3 and claudin-5, ZO-1, and occludin have been described as the predominant proteins found at the TJs [17]. Claudins are crucial for the paracellular sealing function, hence, they directly determine barrier function [18]. However, the presence of claudin-3 in TJs of mouse BBB ECs has been recently called into question: immunodetection of claudin-3 in mouse brain ECs is thought to be an artifact due to antibody cross-reactivity with an un-

disclosed 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 signs of BBB dysfunction [19]. Claudin-5 knockout mice, however, have a significant paracellular 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 conditions [18]. Likewise, disassociation of the ZO-1 junctional complex, which typically serves to stabilize TJs, leads to the breakdown of BBB integrity [22]. The adherens junctions transmembrane components cadherin and nectin anchoring to cytoplasmic components such as catenin and afadin, respectively, are also involved in BBB integrity [23, 24] (Fig. 1).

In addition to the intercellular binding systems that limit paracellular diffusion, cerebral ECs also lack fenestration and exhibit extremely low rates of transcytosis, both of which greatly limit transcellular diffusion [25]. Both features arise from several changes to EC morphology and biochemistry that occur during embryonic development and allow the transition from a permeable to a continuous vessel [26]. 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 TJs preventing the extravasation of molecules from fenestrated capillaries to the parenchyma via the cerebrospinal fluid, thus forming the blood-CSF barrier [see 27 for a review].

The control of substance exchange between blood and cerebral parenchyma is also mediated by specific carrier systems present in EC abluminal or luminal plasma membranes (Fig. 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 family and the solute carrier transporter superfamily [29]. Notably, efflux transporters (e.g., P-glycoprotein, 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

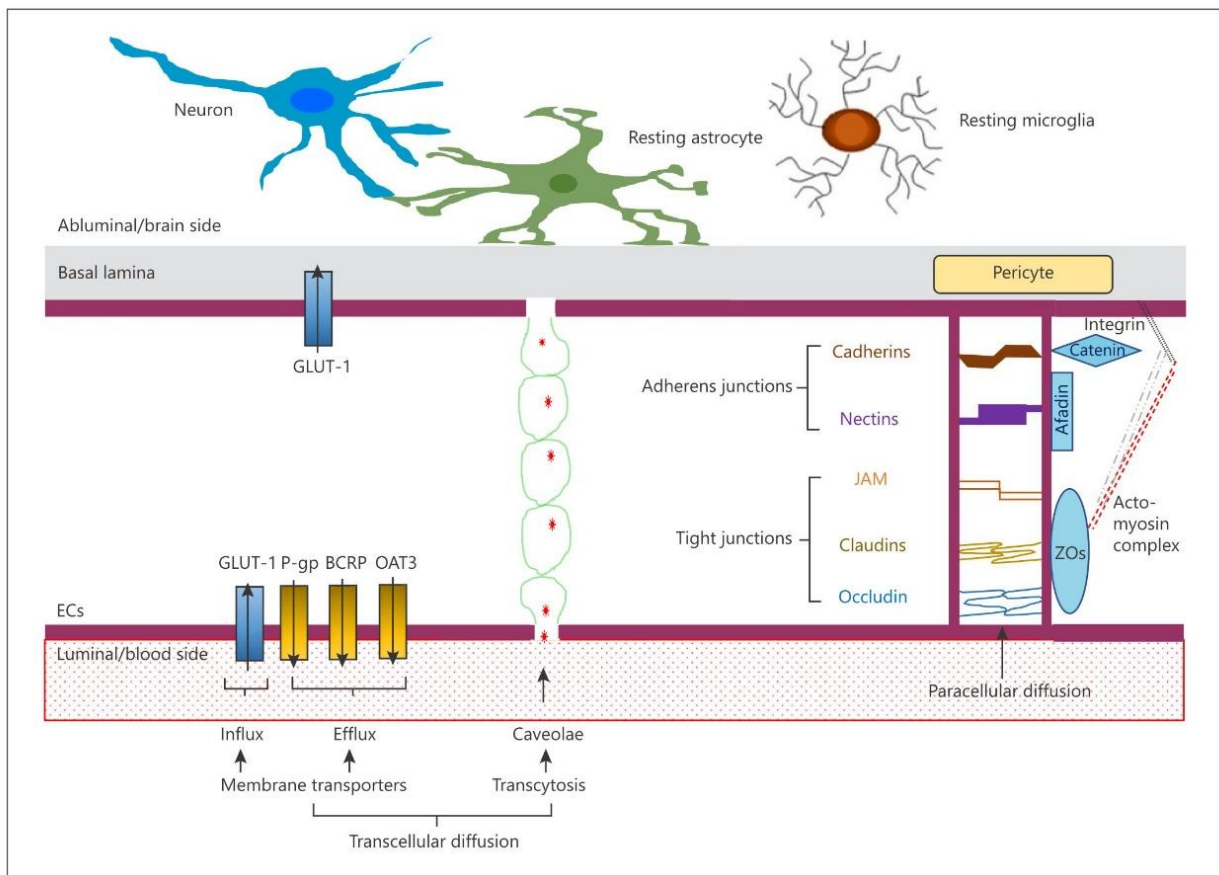


Fig. 1. The major cellular elements of the BBB localized at CNS microvessels are comprised of a continuous layer of ECs, basement membrane cells, pericytes, microglial cells, neurons, and astrocyte end-feet. BBB, blood-brain barrier; BCRP, breast cancer resistance

protein; CNS, central nervous system; ECs, endothelial cells; GLUT-1, glucose transporter 1; JAM, junctional adhesion molecule; OAT3, organic anion transporter 3; P-gp, P-glycoprotein; ZO, zonula occludens proteins.

in the 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 transcellular pathway accounting for BBB permeability in brain injury models [32, 33] (Fig. 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 (Fig. 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 microvessels [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 regulate permeability and vascular remodeling [37, 38], and that they would have contractile capabilities to allow them to regulate blood flow through the capillaries [39]. Moreover, pericytes play a major role in the differentiation of ECs during fetal life, even before the appearance of astrocytes [40]. The recruitment of pericytes to endothelium and the interaction between the two are essential factors for the formation, maturation, and maintenance of the BBB [40–42].

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 glycoproteins [43]. The interaction between endothelial integrin, laminin, and other ECM proteins allows ECs to be anchored onto this basal lamina [44]. In addition to its structural role, ECM degradation is associated with an increase in paracellular permeability of the BBB [45].

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 46 for a review]. When circulation begins to form at the CNS, at a time when the BBB is still permissive, some microglial cell precursors enter the CNS through the vascular network [see 47 for the different hypotheses about the origin of microglia]. Those microglial cells that enter the brain parenchyma during embryonic development then branch out and remain quiescent [48]. 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 TJs of the ECs, the promotion of the enzyme system gamma-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 BBB [51–53].

Dysfunction of the BBB is associated with neurological damage and disease [54]. Disruption of the BBB is also associated with peripheral diseases such as diabetes [55] and nonneural, inflammation-related pathologies [56]. Alteration of the cells of the NVU upon aging, inflammatory activation, oxidative stress, or neuropsychiatric and neurodegenerative diseases leads to BBB dysfunction [44], which, in turn, contributes to the progression of neurological diseases [17, 44].

Testosterone

Testosterone was classically delineated as a male-specific hormone according to its marked synthesis in the testes and its contribution to phenotypic and reproductive sex differences [2].

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 females [57]. Testosterone, which is a precursor of ovarian estrogens, is also present in females, but at lower concentrations (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 disorders [64]. In addition to age-related circulating testosterone changes, there are obvious sex differences in the prevalence of neuropsychiatric (e.g., depression, anxiety disorder, and anorexia nervosa) and neurodegenerative (e.g., cognitive decline, autoimmune diseases, Alzheimer disease [AD], and Parkinson disease [PD]) diseases, with a higher incidence in females than in males [8, 64]. The reasons for sex differences are still unclear, and a better understanding of how the state of being male limits these CNS disorders is still lacking. 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 regulations and pathologies [66].

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 nonreproductive tissues such as cardiac and skeletal muscle, or of the development and function of the immune and nervous systems [1], including cerebrovascular tissue [67–70]. Outside the brain, testosterone has a key effect on the cardiovascular system by regulation of the vascular tone [71]. The greater incidence of clinical manifestations of heart disease in men and postmenopausal women compared with premenopausal women translates a sex steroid hormones-induced sex difference for the vascular tone with a possible vascular

protective effect of female sex hormones. It has been suggested that this sex difference in vascular tone is mediated at least in part by specific sex hormone receptors [68]. Nevertheless, the role of testosterone in vascular contractility is debated. A number of studies have shown that testosterone facilitates vasoconstriction in rats (in vivo and in vitro) [72, 73], while other data have reported a vasodilator effect in rats and rabbits [74, 75]. Vascular mechanisms of testosterone have been thoroughly reviewed very recently [76]. In the brain, the regulation of cerebrovascular items is mediated through several mechanisms such as for instance angiogenesis, vascular contractility, and control of BBB permeability.

Distribution of Sex Steroid Receptors Mediating Testosterone Effects in the Male CNS

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 as well as in neuroendocrine function [2]. 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 (about 0.3%) [1, 77]. The reductase enzyme catalyzes the conversion of about 7% of testosterone to dihydrotestosterone (DHT), which is 2.5- to 10-fold more potent than the former [78, 79]. ER- and AR-mediated signaling pathways modulate several CNS activities by both genomic and nongenomic actions [5, 79]. To describe sex steroid receptor localization within the adult CNS, mRNA expression patterns of ARs and ERs were investigated in adult rats [79–81] (Fig. 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, the bed nucleus of the stria terminalis, and the preoptic area (POA) of the hypothalamus involved in neuroendocrine functions and reproductive behavior [79] (Fig. 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 endothelium [5].

Neural estrogens exert their effects through interaction with two nuclear ERs: ER α and 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 , e.g., at the level of the ventrome-

dial hypothalamic nucleus, while others express only ER β , e.g., paraventricular nuclei. In contrast, other regions like the bed nucleus of the stria terminalis, the medial and cortical amygdaloid nuclei, the POA, as well as the 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] (Fig. 2). It is noteworthy that the highest level of ER α mRNA expression is in the hypothalamus and amygdala and is related to regulation of sexual behavior and the hypothalamic-pituitary-gonadal axis [82].

In addition to their neural expression, specific receptors for gonadal steroids are expressed in the cerebrovascular tissue [5]. 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 CNS

As evoked above, sex 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 remyelination 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 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 the androgenic pathway on myelin repair and remyelination 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

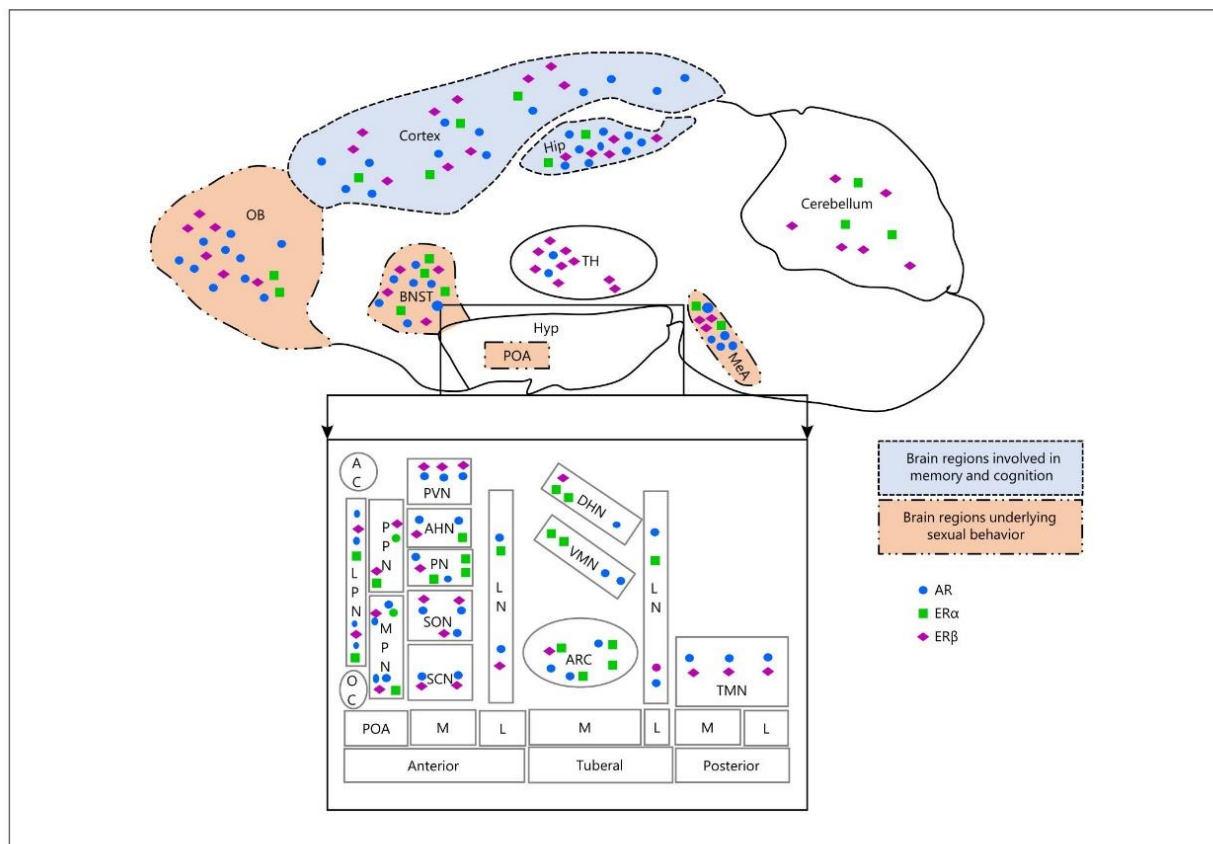


Fig. 2. Overview of a whole male brain with a close-up view of the position of hypothalamic nuclei pointing out the relative location and density of AR (blue circles) and ER (ER α , green squares; ER β , pink diamonds) mRNA distribution using in situ hybridization analysis in a male rat [79–81]. AC, anterior commissure; AHN, anterior hypothalamic nucleus; AR, androgen receptor; ARC, arcuate nucleus; BNST, bed nucleus of the stria terminalis; DHN, dorsomedial hypothalamic nucleus; ER, estrogen receptor; Hip,

hippocampus; Hyp, hypothalamus; L, lateral; LN, lateral nucleus; LPN, lateral preoptic nucleus; M, medial; MeA, medial amygdala; 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, supraoptic nucleus; SON, supraoptic nucleus; TH, thalamus; TMN, tuberomammillary nucleus; VMN, ventromedial nucleus.

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 extranuclear sites of hippocampal neurons [90], has revealed the involvement of testosterone and DHT in neural activity and synaptic function. Gonadectomy also decreases the density of spinal 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 induces 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 [94]. 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 of 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 glucocorticoid 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's dose-dependent neuroprotective and neurodamaging effects in experimental models of cerebral ischemia. Finally, recent experiments in vitro have shown that gonadal hormones may also be involved in the suppression of neuronal apoptosis during ischemic disorders: testosterone and DHT suppress neuronal cell death induced by oxygen-glucose deprivation/reoxygenation in hippocampal slice cultures [103].

In many brain diseases, disorders, traumas, or strokes, neuronal damage is associated with an inflammatory response [104] and a dysfunction of the BBB [105]. However, in a model of multiple sclerosis a partial uncoupling of inflammation and neurodegenerative process has been shown [106]. This study also suggests that 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 in 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 [26] (Fig. 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 [111]. The patterns of circulating levels of testosterone in the male mouse from birth to adulthood are illustrated in Figure 3b [112]. Several researchers have focused their activities on the impact of testosterone on angiogenic mechanisms in cardiovascular regeneration and cerebral angiogenesis in adults [1, 62, 113]. However, the role of testosterone in CNS angiogenesis during fetal life has remained largely unexplored. In cultured human umbilical ECs, testosterone induces EC migration through myosin activation and actin cytoskeleton remodeling [114]. It also increases formation of EC tubes by stimulating Smad1 phosphorylation. This represents one possible mechanism for testosterone-enhanced angiogenesis [115]. 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 [116]. 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 upregulation of VEGF, VEGF receptors, and brain-derived neurotrophic factor [117–119].

Impact of Testosterone on Cerebral ECs and BBB

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 steroids 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 nonmedical purposes, and (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 per-

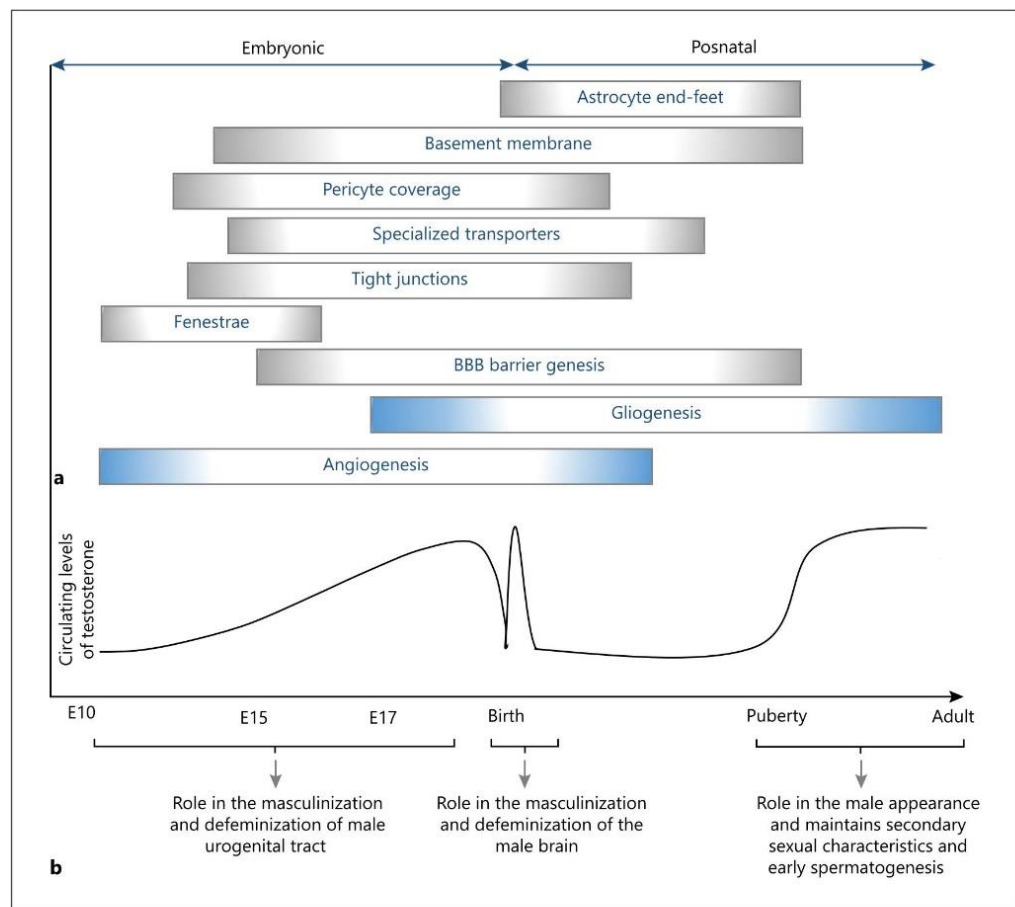


Fig. 3. a Developmental timeline of the BBB setup in the murine CNS [26]. **b** Pattern of circulating levels of testosterone in the male mouse from birth to adulthood [112]. BBB, blood-brain barrier; CNS, central nervous system; E, embryonic days.

fect 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, i.e., brain homeostasis. Testosterone modulates cerebral EC and BBB 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 metabolite [120], is expressed at the BBB [13]. The expression of OAT3 in male rats is higher than in females [121], and ARs regulate the expression and uptake rate of OAT3 in BBB cells (Fig. 4) [83]. It is noteworthy that the dopaminergic system participates not only in sexual behavior processes, but also in regulation of cognitive behavior [122].

Sodium-dependent and -independent 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 BBB [123]. The activity of these transporters is subject to modulation in a variety of neuropathological and neuropsychiatric disorders, including AD, PD [124], and depression [125]. A study proposed

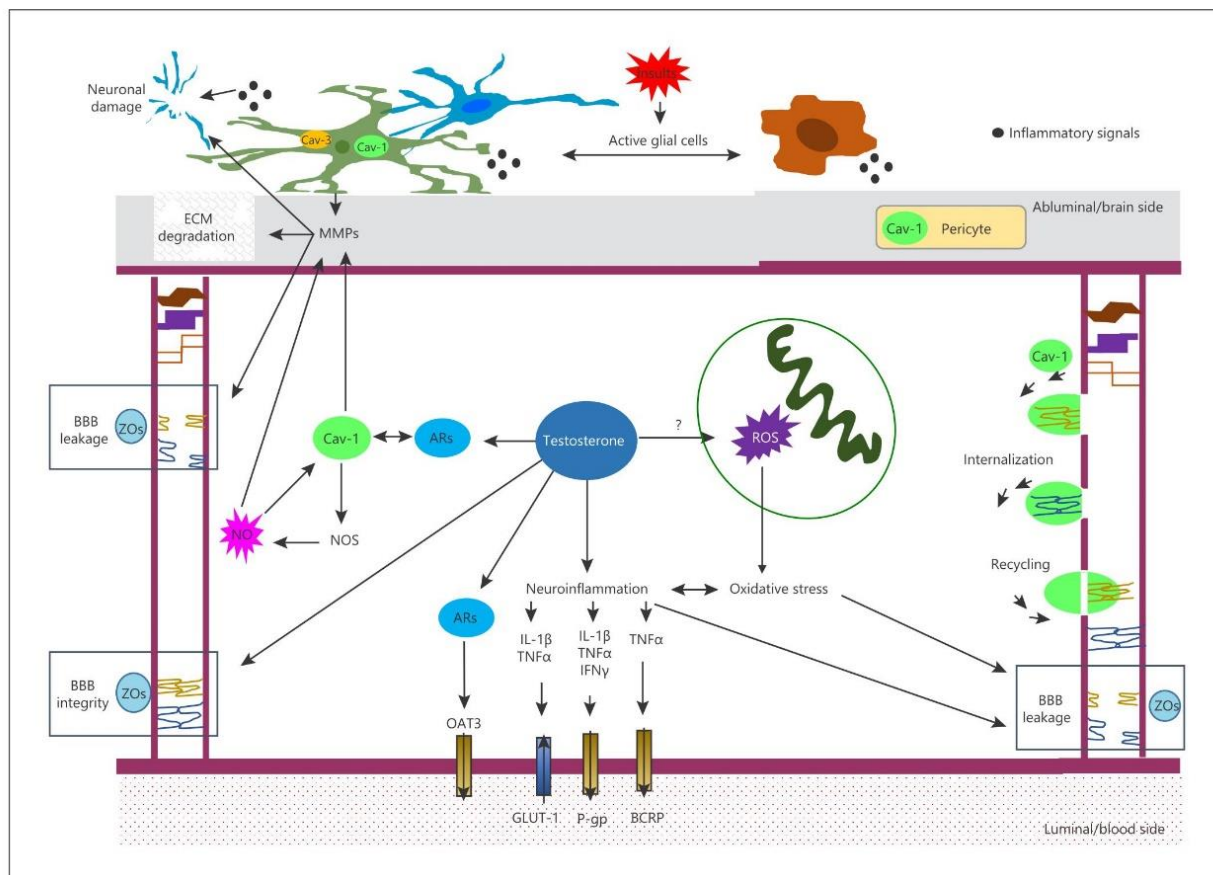


Fig. 4. State of the art schematic representation of testosterone signaling-induced cellular and molecular interactions in the BBB system, highlighting in particular the Cav-1/NO/MMP pathway involvement. Testosterone controls the integrity of the BBB by preserving the intercellular molecular binding protein degradation by regulating endothelial transporter expression or activity and by decreasing inflammatory response. Cav-1 protein is known as an AR coactivator. Cav-1 protein is expressed by brain ECs 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 and (2) inhibition of NO production and consequently inhibition of MMP 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 neuronal loss, degradation of TJ proteins, decrease in the number

of GLUT-1, and decrease in the activity of P-gp and BCRP transporters. Testosterone, in turn, decreases inflammatory mediators. In addition, ARs positively regulate the expression and uptake rate of OAT3. Oxidative stress caused by overproduction of ROS activates inflammatory factors. The cross talk between oxidative stress and neuroinflammation causes BBB leakage. The involvement of testosterone in oxidative stress is not well known. AR, androgen receptor; BBB, blood-brain barrier; BCRP, breast cancer resistance protein; Cav, caveolin; CNS, central nervous system; ECM, extracellular matrix; ECs, endothelial cells; GLUT-1, glucose transporter 1; IFN γ , interferon gamma; IL-1 β , interleukin 1 beta; MMP, matrix metalloproteinase; NO, nitric oxide; NOS, nitric oxide synthase; OAT3, organic anion transporter 3; P-gp, P-glycoprotein; ROS, reactive oxygen species; TJ, tight junction; TNF α , tumor necrosis factor alpha; ZO, zonula occludens proteins.

that proinflammatory cytokines induce protein degradation of the number of glucose transporters, specifically GLUT-1 and GLUT-3, in the BBB of AD patients (Fig. 4) [126]. It is notable that ovarian steroids are essential mod-

ulators of glucose transporters [124, 127]. 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. However, it will not be surprising that testosterone could act as a modulator of influx and/or efflux transporters partly through the modulation of inflammatory mediators. Indeed, the impact of the inflammatory mediators on BBB transporters has been described [128, 129]. For instance, the inflammatory mediators, such as tumor necrosis factor alpha (TNF α), interleukin 1 beta (IL-1 β), and interferon gamma can decrease the activity of P-glycoprotein at the level of the BBB (Fig. 4) [130]. P-glycoprotein contributes to the efflux of brain-derived amyloid into blood. In addition, TNF α reduces the functional activity of breast cancer resistance protein (Fig. 4) [131], 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 upregulation of IL-1 β and TNF α in the POA [67] (Fig. 4), although the effects of these shifts upon membrane transporters were not evaluated.

Transendothelial Vesicular Transport Pathway

As already mentioned, caveolae are known as a major transcellular pathway in brain injury models. It was shown that the components of the caveolae plasma membranes, Cav proteins, play a critical role in signal transduction [77]. In addition to brain ECs, the Cav-1 protein has been described in vascular pericytes [132], while both Cav-1 and Cav-3 have been found *in vivo* and *in vitro* in reactive astrocytes (Fig. 4) [132, 133].

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 upregulation of the expression level of Cav-1 [34]. Nonetheless, data obtained from Cav-1 and Cav-2 knockout mice have shown a higher vascular permeability and a lower lesion volume, respectively, after a stroke compared to wild-type mice [134]. This suggests a positive role of Cav-1 in the maintenance of the integrity of the BBB in contrast to Cav-2. Cav-1 knockout ischemic brains are also characterized by impaired angiogenesis and increased cell death [134]. The role of Cav-1 in BBB integrity in the context of neuropathology and neural recovery after ischemic stroke was reviewed very recently, and the discrepancies in the literature pertaining to the role of Cav-1 were discussed [135].

Data based on rat immunohistochemistry analyses showed that an increase in Cav-1 expression at the cortical lesion site of a cold injury blood vessel model is associated with loss of occludin and claudin-5 TJ proteins [136]. However, it has been suggested that Cav-1 expression con-

tributes 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 inflammation [137]. Recycling of internalized TJ proteins is crucial for recovery of the brain endothelial barrier integrity [137] (Fig. 4).

Despite confusion in the current literature concerning the roles of Cav-1 in the modulation of BBB integrity, thus far studies have provided evidence that Cav-1 could prevent oxidative and inflammatory injury as well as extracellular degradation of TJ proteins and matrices [137, 138]. 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 nongenomic 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, phosphoinositide 3-kinase, and protein kinase B [77]. Cav-1 protein is known as ARs coactivator [77, 139], allowing this nuclear receptor to efficiently mediate gene expression. Thus, it cannot be excluded that AR nongenomic 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 AR transactivation and sensitivity of ARs to ligand-dependent activation, confirming the role of Cav-1 as a convergent point for AR cross talk with other cellular signal transduction pathways (Fig. 4) [140]. 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 AR requires further investigation.

Basal Lamina and the Immediate Surroundings of ECs

The basal lamina as an acellular membrane surrounding ECs has a pivotal role in BBB integrity [141]. Loss of basal lamina stability triggered by metalloproteases or collagenases is frequently observed in pathological manifestations involving BBB breakdown [142].

Nitric oxide (NO) is a diffusible gas which at 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 pathways [143]. The basal concentration of NO derives from endothelial NO synthase (eNOS) enzyme activity, whereas deleterious effects are due to the activity of neuronal (nNOS) and inducible NOS (iNOS) isoforms [143].

NO mediates its physiological effects at least in part by the activation of matrix metalloproteinases (MMPs) during matrix reorganization [143]. MMP2, MMP3, and MMP9 are the main forms found in the brain and are the prime proteolytic enzymes particularly involved in the degradation of the ECM [144] and in neuronal damage [145–147]. Activated MMPs also lead to BBB leakage by catalyzing hydrolysis of the TJ proteins, thereby inducing their degradation [147–149] (Fig. 4). In addition, both MMP2 and MMP9 contain Cav-binding motifs, and there is direct evidence that Cav-1 can downregulate MMP activities and decrease BBB permeability in brain microvascular ECs during cerebral ischemia-reperfusion injury [150]. 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 (Fig. 4) [150]. In a recent investigation, the Cav-1/NO/MMP pathway has been suggested as a therapeutic target to ensure protection against cerebral ischemia-reperfusion injury (Fig. 4) [138]. 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 Cav-1/NO/MMP pathway, ensuring BBB integrity.

Pericytes are located around the cerebral capillaries, and they share the basal lamina with ECs. Despite a decrease in pericyte numbers and ultrastructural changes in aging and age-related brain diseases such as AD, in both human and preclinical models [151], there are no data concerning the effects of sex steroid hormones on pericytes. Further investigations in this regard are clearly required.

Interendothelial TJs

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 [26, 44]. 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 multiple sclerosis [44], as well as in physiological aging [13].

It is now well known that ovarian hormones influence BBB integrity with respect to limiting paracellular permeability via the preservation of TJ proteins [69, 152]. However, the contribution of testosterone to the BBB TJs response is poorly defined.

The first conclusive evidence that under physiological conditions testosterone impacts TJ protein expression in males was obtained in our 2017 study [67]. This showed that castration of adult male mice significantly decreased the expression of TJ proteins (claudin-5 and ZO-1 but not occludin) in the hypothalamic POA, 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. Supplementation with testosterone after castration restores BBB impermeability and TJ 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 [153]. Thus far, these results suggest that androgenic signaling supports BBB integrity. However, this study does not shed light on whether the genomic or nongenomic steroid pathway mediates androgen effects.

Testosterone and Neuroinflammation: Relationships with BBB 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. In-

terestingly, testosterone does not only regulate the development and function of the immune system, but also modulates 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 [154, 155]. Microglia express a cytoplasmic marker, the ionized calcium binding adapter molecule 1 protein, which is upregulated during microglial activation [156, 157]. The degree of activation of microglia appears to be correlated with the type and severity of the brain disorder [158]. 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 activation [159]. Microglia are known as the source of proinflammatory cytokines (e.g., TNF α , IL-1 β , IL-2, IL-4, and IL-6), prostaglandins, chemokines, enzymes associated with inflammation (e.g., iNOS and COX-2) as well as inflammatory cytokines production [160]. By releasing cytokines such as TNF α , activated microglia induce neurotoxic reactive astrocytes (termed “A1”) which might have harmful functions [161]. Numerous studies indicate that following CNS injury, reactive astrocytes upregulate both vimentin and glial fibrillary acidic protein [162, 163]. Astrocyte-inducible NOS has been described to play a major role in human neuroinflammation [164–166].

Gliosis corresponds to the reactive change of glial cells (microglia and astrocytes) in response to damage [167, 168]. This is conjugated with the modulation of local chemokines and cytokines, which govern inflammation (Fig. 4). Chronic gliosis may heighten the neurodegenerative process. Thus, neuroglia have been identified as a therapeutic target to halt inflammation in several neurodegenerative diseases [169].

The action of testosterone on reactive gliosis is not well documented yet. However, available evidence indicates that administration of testosterone, but not DHT, down-regulates reactive gliosis after brain injury in male rats [110, 170, 171]. Recently, it has been shown that reactive astrocytes induce BBB disruption at least in part via the production of VEGF-A and thymidine phosphorylase in the case of inflammatory brain lesions [172].

Furthermore, we have shown that castration of male mice is associated with activation of neuroglial cells. This occurs via upregulation of inflammatory proteins such as COX-2 and iNOS as well as proinflammatory

cytokines such as TNF α . Interestingly, supplementation of testosterone after castration suppresses these inflammatory features [67]. 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 proinflammatory molecules such as TNF α and IL-1 β [173]. In addition, it has been proposed that in turn, BBB dysfunction contributes to the progression of neurodegenerative diseases [14]. 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 BBB Dysfunction

The sensitivity of the BBB to oxidative stress is due to a high mitochondrial content (8–11% of the endothelial cytoplasmic volume) in cerebral ECs, which is pivotal to maintain active transport [174]. 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 ROS [175] (Fig. 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 inflammatory factors [176]. 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) [177]. In addition, oxidative stress induces inflammation, which can further increase oxidative stress activity (Fig. 4) [178]. Low testosterone levels [179, 180], aging [181], and testosterone deprivation [182] are risk factors for oxidative stress. It was shown that there is an inverse association between testosterone concentrations and oxidative stress markers in adult men [183]. 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 homeostasis [184]. To date, the involvement of testosterone in oxidative stress at the level of the NVU is still not well established and needs further investigation. The effects of testosterone upon oxidative status are still controversial, ranging from ROS generation to scavenging or chain breaking of ROS and RNS [185–188]. Numerous studies have indicated that the effect of testosterone in this regard is tissue-, sex-, and dose-dependent [189]. 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 [190]. Moreover, it was shown that in these cell lines testosterone causes ROS and H₂O₂-induced apoptosis [191]. Like cerebral ECs, 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 apoptotic processes [192]. However, it has been reported that androgens induce ROS generation through AR signaling in cultured vascular smooth muscle [193] and renal interlobar arteries [194].

Conclusion

The aim of this review was to present an overview of the literature data concerning the effects of testosterone on the NVU in males. It mainly emerges from these studies that (a) testosterone promotes cerebral angiogenesis and vasculature formation, (b) testosterone supports the integrity of BBB TJs, and (c) testosterone regulates some endothelial membrane transporters, although very few data are available yet. The effects of testosterone are mediated by either androgen or ERs 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 the Cav-1/NO/MMP pathway.

Dysfunction of the BBB is often associated with an inflammatory status inducing a neurodegenerative process. However, the link between neuroinflammation and disruption of the BBB is still poorly understood. Data sug-

gest that testosterone could prevent from gliosis reaction and upregulation of inflammatory proteins and could participate in reducing ROS generation and limiting oxidative stress.

To conclude, data from the literature show an undeniable role of testosterone on brain capillaries guaranteeing 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 BBB integrity and the associated signaling pathways remain to be analyzed in greater detail.

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 partly answer the enigma of sex differences and of 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 pathways and thus exert potentially deleterious effects on health. Considering the abundance of environmental contaminants that have antiandrogenic and/or antiestrogenic properties, consideration should be given to the effects of these components on brain microvessels.

Acknowledgments

We thank Dr. Sakina Mhaouty-Kodja (Neuroscience Paris-Seine, Sorbonne University, Paris, France) for attentive reading and pertinent advice.

Conflict of Interest Statement

The authors declare no competing interests with respect to the research, authorship, and/or publication of this article.

Author Contributions

D. Ahmadpour wrote the manuscript with significant input from V. Grange-Messent.

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