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Review

Neurovascular adaptations modulating cognition, mood, and stress responses

Laurence Dion-Albert [1,4], Katarzyna A. Dudek [1,4], Scott J. Russo [2], Matthew Campbell [3], and Caroline Menard [1,*]

The neurovascular unit (NVU) is a dynamic center for substance exchange between the blood and the brain, making it an essential gatekeeper for central nervous system (CNS) homeostasis. Recent evidence supports a role for the NVU in modulating brain function and cognition. In addition, alterations in NVU processes are observed in response to stress, although the mechanisms via which they can affect mood and cognitive functions remain elusive. Here, we summarize recent studies of neurovascular regulation of emotional processes and cognitive function, including under stressful conditions. We also highlight relevant RNA-sequencing (RNA-seq) databases aiming to profile the NVU along with innovative tools to study and manipulate NVU function that can be exploited in the context of cognition and stress research throughout development, aging, or brain disorders.

Highlights

The neurovascular unit (NVU) has been long recognized as an interface for substance exchange between the blood and the brain. However, beyond these canonical roles, cells of the NVU contribute to cognitive processes, including learning, memory, and behavioral flexibility.

Alterations of attention allocation in stress-related pathologies could be mediated, in part, by astrocyte dysfunction and neurovascular inflammation.

Interventions reducing chronic stress-induced blood–brain barrier dysfunction may promote resilience and mitigate associated cognitive impairments.

Emerging viral, transgenic, and transcriptomic tools enable precise and selective investigation of the involvement of NVU cells in cognition, both in health and disease.

Cognition and affective behavior, beyond neurons

Cognition involves a wide variety of processes related to the acquisition, storage, manipulation, and retrieval of information to appropriately perceive and interact with the world. Fundamentally, cognition involves thoughts and behaviors, including highly complex processes, such as memory, attention, and mental flexibility. Constantly evolving and adapting to new information across lifespan, cognition is shaped by genetic and environmental factors, among others. Prolonged stress exposure induces dysregulation of stress coping mechanisms and cognitive functions that can lead to mental illnesses, such as anxiety disorders and major depressive disorder (MDD) [1–3], which are often comorbid with other neurological conditions, for instance attention deficit/hyperactivity disorder (ADHD) [4] and neurodegenerative diseases [5]. Much of what is known about cognition relates to how neurons encode information to support cognitive processes. However, research studying astroglia, endothelial cells, and other non-neuronal cells has unraveled functions beyond the facilitation of neuronal activity to a more central and active role in cognition and behaviors [6–8].

The NVU is a highly complex and heterogeneous system (Figure 1). A growing body of research in neuroscience, neurology, and psychiatry is examining how the NVU coordinates and modulates brain activity to regulate cognition over diverse timescales [9]. Much of these advances result from the advent of new tools to target endothelial cells, astrocytes, and mural cells, and have led to the discovery of mechanistically novel pathways and functions. In this review, we first describe recent findings on the neurovascular regulation of behavior, with a specific focus on how stress alters cognitive functions, such as memory, attention, and behavioral flexibility. These findings are contextualized through the lens of chronic stressors and implications for human neuropsychiatric conditions. We then discuss the advantages and limitations of a new generation of tools that target neurovascular cells precisely and selectively, concluding with a discussion of their relevance for human translational research.

[1]Department of Psychiatry and Neuroscience, Faculty of Medicine and CERVO Brain Research Center, Université Laval, Quebec City, QC, Canada
[2]Nash Family Department of Neuroscience, Icahn School of Medicine at Mount Sinai and Center for Affective Neuroscience, 1 Gustave L Levy Place, New York, NY, USA
[3]Smurfit Institute of Genetics, Trinity College Dublin, Lincoln Place Gate, Dublin 2, Ireland
[4]These authors contributed equally.

*Correspondence:
caroline.menard@fmed.ulaval.ca
(C. Menard).

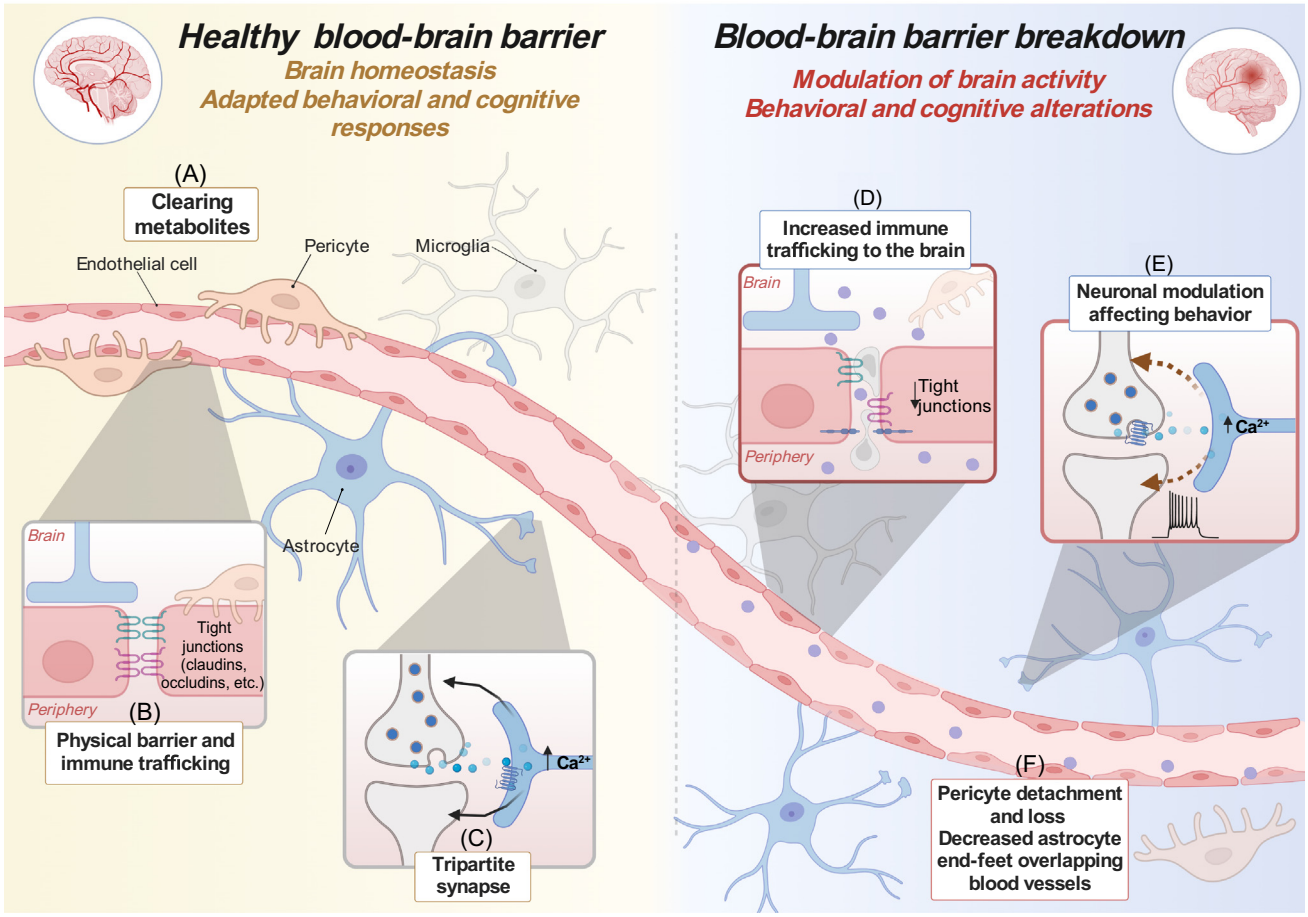

Figure 1. Cells of the neurovascular unit (NVU) modulate brain activity and cognition in health and diseases. The blood–brain barrier (BBB) comprises endothelial cells (pink), astrocytes (blue), pericytes (yellow), and microglia (gray) [9]. The main roles of the BBB are to: (A) maintain brain homeostasis through clearance of metabolites and toxins; and (B) provide a physical barrier preventing entry of harmful molecules from the periphery into the brain and regulate immune trafficking. Astrocytic processes are found ensheathing the pre- and postsynaptic neuronal components of synapses, forming the tripartite synapse (C). Release of neurotransmitters activate G-protein-coupled receptors (purple) on the astrocyte end-feet, stimulating Ca^{2+} release from intracellular stores regulating pre- and postsynaptic currents, and subsequently cognitive processes [11,12,58]. BBB breakdown, a hallmark of several stress-related disorders and brain diseases, affects cognition through different NVU-related mechanisms [28,30]. For example, increased peripheral inflammation leads to vascular dysfunction and loss of tight junction proteins as well as enhanced immune trafficking to the brain (D) [2,3]. Moreover, altered astrocyte states at the tripartite synapse can affect modulation of synaptic currents and cognition-related neuronal networks (E) [8,12]. Finally, pericyte detachment and decreased coverage of blood vessels by astrocyte end-feet further contribute to BBB breakdown [22], trafficking of immune cells and inflammatory mediators into the brain and altered neuronal signaling [23], potentially leading to behavioral and cognitive dysfunction (F). Figure created using BioRender (www.BioRender.com).

NVU homeostasis and signaling in the healthy brain

The brain is the most metabolically active organ of the body, consuming in humans ~20% of oxygen/glucose at rest for only 2% body weight. It is supplied by a complex network of blood vessels maintaining homeostatic balance, clearing undesirable metabolites, and ensuring immune trafficking [10] (Figure 1A,B). Endothelial cells forming blood vessels are the first frontier between the periphery and the brain, and the interendothelial space of the neurovasculature is sealed by tight junction complexes (Figure 1B) [2,3,9]. Structural integrity of the vascular wall is further supported by mural cells, including smooth muscle cells and pericytes, which surround brain endothelial cells [10]. Astrocytes are non-neuronal cells that regulate synapses, neuronal circuits, and behaviors [11]. They are found ensheathing neuronal connections, forming the tripartite synapse

(Figure 1C) [12–14]. Microglia, the brain-resident immune sentinels, are not part of the NVU but remain close to the vasculature, notably in areas lacking astrocyte end-feet [15]. They sense, monitor, and regulate neuronal activity in both physiological and pathological conditions, and can modulate behaviors [16]. NVU cells maintain blood–brain barrier (BBB) integrity and the tightly regulated microenvironment of the brain ensuring CNS homeostasis and preventing passage of circulating inflammation (Figure 1D).

Local increases in cerebral blood flow (CBF) in response to neuronal activity are described as neurovascular coupling, and are possible due to highly coordinated crosstalk among neurons, astrocytes, mural cells, and endothelial cells [8]. Neurons can release vasoactive compounds that act directly on vascular smooth muscle cells (VSMCs) to promote vasomodulation in response to metabolic demands. Astrocytes are well positioned to sense and respond to both neuronal and vascular activity (Figure 1E). Indeed, they orchestrate neurovascular coupling by sensing neuronal activity, which triggers calcium signaling that propagates to their end-feet to promote the release of vasoactive factors and subsequent dilatation of VSMCs to increase capillary perfusion [17]. Moreover, astrocytes assemble the basement membrane though laminin expression, critical to maintain BBB function. Astrocyte-specific knockout of laminin in mice leads to increased BBB permeability and impairment of NVU coupling through impaired VSMC function [18]. This cell type is highly heterogeneous, with several distinct subtypes currently identified [19,20], but the functional roles of each of these subtypes in the context of neurovascular coupling and behavior remain to be elucidated. Endothelial cells also participate in neurovascular coupling by expressing potassium channel $K_{IR}2.1$, rendering them sensitive to changes in neuronal activity [21]. Finally, pericytes regulate the development and maintenance of BBB integrity [22], while also contributing to neuronal function. Indeed, ablation of pericytes leads to reduced CBF, BBB hyperpermeability, and neuronal loss via depletion of the neurotrophic growth factor pleiotrophin [23] (Figure 1F).

Much of the current knowledge regarding the disruption of NVU integrity and function and its impact on cognition comes from studies related to aging, neurodegenerative disorders [e.g., Alzheimer's disease (AD) and vascular dementias], stroke, or traumatic brain injury (TBI) [24–32]. This body of work outlines a path between cognitive deficits and NVU dysfunction, particularly via impaired CBF, disrupted neurovascular coupling, oxidative stress, and BBB hyperpermeability, facilitating infiltration of circulating inflammatory mediators into the brain (Figure 2). However, whether similar mechanisms are involved in stress-related illnesses, such as MDD, which is also characterized by cognitive impairments [33] and often exacerbated inflammation [34], is unclear. We propose that cognition and behavior are intrinsically linked to the proper regulation of vascular homeostasis and NVU coupling, and that chronic stress can hamper these systems, leading to inappropriate behavioral regulation and mood disorders.

NVU in the context of cognitive function and stress responses

Stress and memory

Memory is defined as the ability to retain perceived and/or learned information. Memories can be classified based on their duration and capacity. One of the prevalent classifications is into short-term versus long-term memory. Short-term memory, generally lasting up to 1 min, temporarily stores a limited amount of information, which is then applied to perform a cognitive task in the process known as working memory [35]. Ranging from hours to years, the storage capacity of long-term memory is large, with no known limit. Formation of long-term memories, enabling the organism to benefit from previous experiences and properly adapt to stimuli, is essential for survival. In terms of underlying biological mechanisms, short-term and long-term memory differ, with post-translational protein modifications being associated with the former, and *de novo* gene expression with the latter [35].

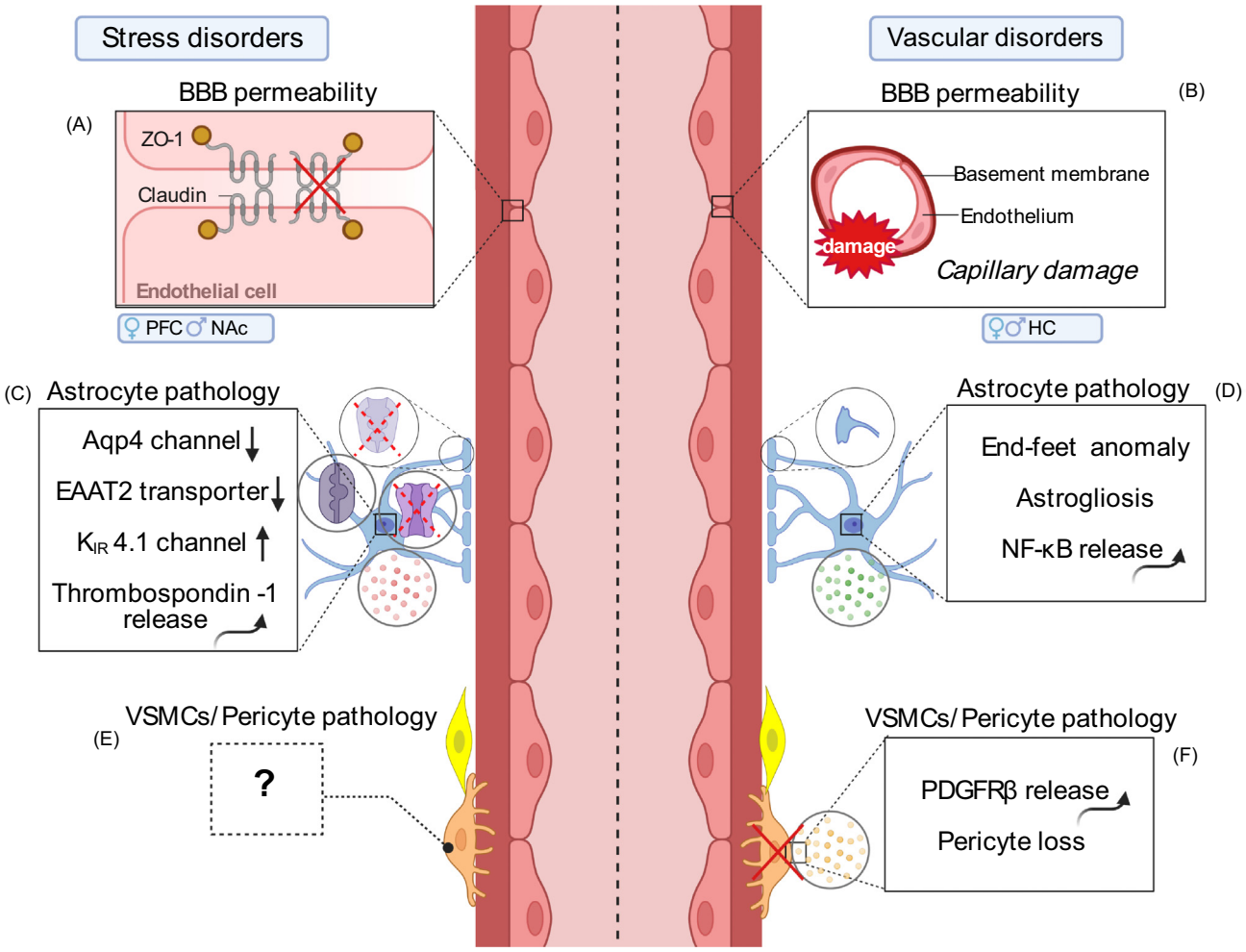

Figure 2. Comparison of neurovascular unit (NVU) alterations between stress and cerebrovascular disorders. Blood–brain barrier (BBB) leakiness, provoked by stress-induced downregulation of the tight junction protein Claudin 5 (Cldn5), is observed in the prefrontal cortex (PFC) of female mice and in the nucleus accumbens (NAc) of male mice. Loss of CLDN5 was also noted in the same brain regions in men and women with major depressive disorder (MDD) (A) [45–47]. Hippocampal capillary dysfunction resulting in pathological increase in BBB permeability is an early biomarker of human cognitive dysfunction (B) [25]. Astrocytes can influence neuronal activity via multiple mechanisms. Downregulation of astrocytic excitatory amino acid transporter (EAAT) 2, which is associated with disruption of glutamate uptake, and increased K_{IR}4.1 channel expression occur in preclinical models of MDD and postmortem MDD brain samples (C) [48,49,83,91,92]. Decreased vascular coverage by astrocytic end-feet and reduction in perivascular protein aquaporin (Aqp)-4 is observed in human MDD and rodent stress models [83,93], while release of astrocyte adhesive protein thrombospondin 1 is associated with depression in women (C) [73]. Astrocytic end-feet anomalies are also present in vascular disorders characterized by cognitive impairments [24]. These conditions are characterized by activation of white matter astrocytes and release of proinflammatory mediator nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (D) [32]. Vascular smooth muscle cells (VSMCs) and pericytes control interactions between neurons and the cerebral vasculature [22,23]. It remains unclear whether they contribute to stress responses or mood disorders (E). Pericyte loss and disruption in platelet-derived growth factor (PDGF)-BB:PDGF receptor-β signaling have been observed in Alzheimer's disease (F) [31]. Figure created using BioRender (www.BioRender.com). Abbreviation: HC, hippocampus.

Both acute and chronic stress profoundly affect hippocampus-dependent memory, but result in opposite effects on cognitive function [1]. Short-term episodes of mild-intensity stress can facilitate cognitive encoding and working memory performance. By contrast, exposure to high-intensity stressful episodes can lead to impairment of the formation and retrieval of memories and complex cognitive processes, including executive function (i.e., decision making and mental flexibility) [1,36]. Of note, the type and timing of the stressor are crucial. In humans, exposure to

stress or glucocorticoid treatment directly after learning can enhance memory consolidation [37]. Conversely, stress or glucocorticoid administration shortly before memory testing diminishes memory retrieval [38]. Stress elevates catecholamine and glucocorticoid signaling cascades to modulate memory strength [36]. Increases in glucocorticoids favor integration of input from several areas, such as the amygdala and prefrontal cortex (PFC), to regulate emotional memory and cognitive control [39]. Astrocytes express glucocorticoid receptors and, hence, are sensitive to stress hormone release. They can also influence memory processes in a variety of ways, including large-scale coordination of neuronal activity via release of gliotransmitters, control of glutamate reuptake, as well as regulation of CBF, glucose supply, and metabolism [40,41]. In the rodent neocortex and hippocampus, acute stress impairs astrocyte–neuron lactate shuttle, crucial for learning and memory processes (Figure 3A) [42].

Other elements of the neurovascular unit have been implicated in the regulation of memory function. For example, polymorphisms and differential methylation patterns of the endothelial tight junction protein Claudin 5, essential to maintain BBB integrity and function, in the human dorsolateral PFC are associated with cognitive decline (Figure 3B) [43,44]. Stress exposure in mice and human MDD are associated with Claudin 5 loss [45,46]. Claudin 5 transcriptional repression is concomitant with BBB hyperpermeability [47], indicating that epigenetic mechanisms at the NVU could underlie cognitive trajectory, and that stress may potentiate memory deficits via BBB-related pathways. In preclinical models, activation of stress-related neuroendocrine signaling impairs neurovascular coupling by decreasing reactivity of hippocampal arterioles, possibly through $K_{IR}2.1$ channel dysfunction, thus affecting memory (Figure 3C) [48,49]. Neuroendocrine stress blunts proper nitric oxide-mediated vasodilatation of hippocampal arterioles. This effect is sex specific, with arteriolar dysfunction being linked to greater memory impairment in male than in female rats [49].

These studies indicate that components of the NVU contribute to learning and memory processes. Still, more has to be done to uncover how NVU cells regulate memory function (see Outstanding questions). While elegant work underscored a role for endothelial and vascular mural cells in altered vasomotor reactivity/cerebral perfusion and memory dysfunction in neurodegenerative diseases [50] or age-related cognitive decline [25], the potential involvement in MDD-associated learning and memory deficits [33] remains to be determined.

Stress and cognitive flexibility

Behavioral flexibility is a top-down process enabling shifting between rules, tasks, and strategies [51,52]. It is greatly influenced by stress, with acute stressors increasing preservative errors. In humans, cognitive flexibility in response to acute stress is influenced by the subjective perception of participants' chronic stress levels [51], highlighting the importance of both stress modalities in cognition research. Alteration of cognitive flexibility is observed in various conditions, notably schizophrenia [53] and AD [54]. The complex regional and neural correlates underlying mental flexibility in humans and rodents have been elegantly reviewed [54]. Among them, PFC networks projecting to limbic areas were characterized with distinct spatial and temporal effects of stress [51,55].

At the cellular level, reductions in astrocyte number in the rat medial PFC are associated with impaired cognitive flexibility in the attentional set-shifting task [56]. Experiments to examine the underlying mechanism revealed reduced neuronal activity across multiple frequency ranges [56]. Conversely, activation of astrocytic calcium (Ca^{2+}) signaling and Ca^{2+}-binding protein S100β was sufficient to rescue cognitive flexibility [56]. This implies the necessity of astrocytic function to regulate neuronal oscillations and coupling in cognitive flexibility tasks. These effects appear

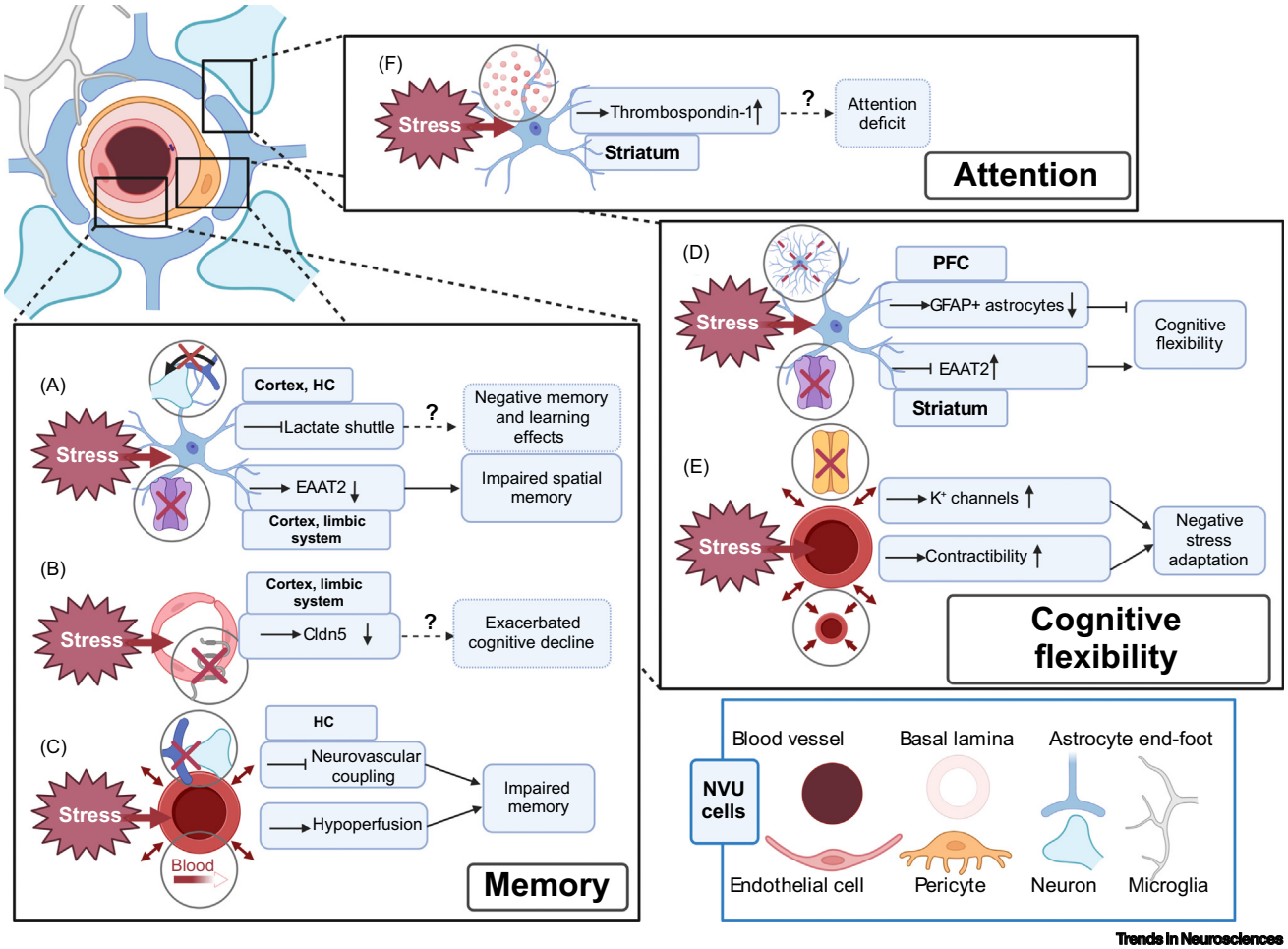

Figure 3. The neurovascular unit (NVU) in the lens of cognitive function and stress responses. Can stress-induced neurovascular pathology negatively affect neuronal function or even contribute to cognitive impairment? Acute stress hinders lactate shuttle between cortical and hippocampal (HC) neurons and astrocytes, a mechanism crucial for memory processes (A) [42]. Disruption of glutamate uptake due to stress-induced astrocytic excitatory amino acid transporter (EAAT) 2 downregulation could contribute to spatial memory impairments (A) [83,86,87]. Chronic stress promotes blood–brain barrier (BBB) leakiness through downregulation of the tight junction protein Claudin 5 (Cldn5) in the cortex and limbic system in a sex-specific manner [45–47]. Curiously, epigenetic *CLDN5* regulation is associated with exacerbated cognitive decline (B) [43,44]. Sex-specific memory impairment has been linked with perturbations in neurovascular coupling, increased vascular contractility, and decreased blood flow in rats (C) [49]. Relatedly, in the prefrontal cortex (PFC) of female rodents, stress leads to astrocyte atrophy and inhibition of cognitive flexibility (D) [60]. Downregulation of striatal EAAT2 caused by stress exposure could negatively affect behavioral flexibility, which is promoted by increases in transporter expression (D) [59]. Negative chronic stress adaptation has been associated with increased expression of voltage-gated potassium (K+) channels and resulting vascular hyperperfusion (E) [65]. Astrocytic thrombospondin 1 release, associated with depression pathology in females, can affect attention (F) [6,73]. Figure created using BioRender (www.BioRender.com).

to be mediated by glutamatergic signaling and are in line with suggested regulation of glutamate extracellular levels via astrocytic receptors [57,58]. In mice, upregulation of astrocytic excitatory amino acid transporter (EAAT) 2 in the striatum resulted in loss of behavioral flexibility. Conversely, preventing EEAT2 upregulation normalized behaviors, suggesting a role for astrocyte-mediated signaling in cognition (Figure 3D) [59]. Rodents exposed to chronic stress exhibit altered glutamatergic receptor densities and astrocyte dystrophy in the PFC, along with deficits in behavioral flexibility (Figure 3D) [60,61]. Thus, astrocyte-mediated signaling in cortical and striatal neuronal networks could contribute to behavioral flexibility both at baseline and under stress. However, additional mechanistic insights are needed. Inflammation, a hallmark of stress disorders, affects

astrocyte function and regulation of neuronal networks [62], and recent evidence suggests that overexpression of major histocompatibility complex I in astrocytes of the mouse PFC can alter discrimination learning [63]. Although this mediator can affect stability of neuronal circuits in several pathologies [64], its mechanistic relevance in astrocytes or inflammation in disease-induced loss of behavioral flexibility remains to be examined (see Outstanding questions).

Behavioral flexibility under chronic stress might also be regulated by other NVU cells. For instance, positive stress adaptation following chronic mild stress in rats, as indicated by a lack of anhedonia for voluntary sucrose consumption, is linked to biphasic neurovascular responses to neuronal activation via changes in arterial contractility (Figure 3E) [65]. Anhedonia, a core symptom of MDD, is defined by reduced motivation and inability to experience pleasure. While no significant changes in $K_{IR}2.1$ channels were found between groups, reduced expression of voltage-gated potassium channel $K_V7.4$ was found in cerebral arteries of hedonic rats (Figure 3E) [65], suggesting an active role for neurovascular integrity in mediating reward responses and possibly behavioral flexibility under chronic stress conditions.

Stress and attention

A bidirectional relationship exists between stress and attention. First, stress modulates attention allocation, and exposure to acute stressors can increase vigilance and attention [66]. However, chronic stress or high trait anxiety can impair attentional control and requires larger cognitive resource allocation to complete tasks [67]. Decreased ability to concentrate is a diagnostic criterion for MDD [68]. ADHD is highly comorbid with psychiatric conditions [69] and overlapping symptomatology implies potentially shared pathophysiology. Anatomical and neuroimaging studies suggest that attentional capacities are underpinned by the frontoparietal control network, a complex and functionally connected circuit managing goal-directed attention and executive control [68,70]. This circuitry encompasses several brain regions, including the PFC [68,70].

Astrocytes provide a structural and modulatory role for neurons in reward–attention circuits, although underlying mechanisms remain to be fully elucidated [71]. Recent work revealed that latent synaptogenic cues derived from striatal astrocytes drive behavioral hyperactivity and disrupted attention in adult male mice [72]. This elegant study provides strong evidence of alternate mechanisms beyond GABAergic medium spiny neuron–astrocyte signaling. Here, astrocytes modulate synapse formation and excitatory synaptic transmission via thrombospondin 1, an adhesive glycoprotein that can affect attentional capacities [72]. This astrocyte-secreted factor is involved in synaptogenesis and regulation of BBB permeability and has been implicated in depression in women (Figures 2C and 3F) [73], suggesting a potentially relevant mechanism through which astrocytes could affect cognition and attention in psychiatric illnesses.

NVU regulation of CBF supply appears to directly contribute to attentional processes in humans. A correlation between ADHD and cerebrovascular perturbations has been reported [74,75], with alterations in both cerebral perfusion and functional connectivity observed in the amygdala of adults with ADHD [73]. Local neuronal activity is often associated with an increase in blood oxygenation and hemodynamic response [76], exacerbated when a cognitive task is performed [77]. ADHD symptoms were correlated with hypoperfusion in the left amygdala, hippocampus, and striatum [73], three major hubs for emotion regulation. Alterations in regional CBF were also reported in these limbic areas in stress-related disorders (reviewed in [3]), suggesting a link, albeit indirect, between NVU coupling, attention, and emotional processing. Vascular alterations were observed in a rat model involving attention dysregulation, which can be seen as manifesting ADHD-like behaviors [78]; the use of this and other animal models could help gain mechanistic insights into the role of the BBB in attentional processes.

Altogether, this body of work supports a role for the NVU in mediating attention and stress-related changes in attentional capacities. It emphasizes the need to think outside of neuronal networks when investigating the pathophysiology of stress- and cognition-altering disorders. Astrocyte-related processes, such as release of synaptogenic cues or maintenance of neurovascular integrity, could be explored for innovative cognition-modulating therapies. Further deciphering the common biology underlying stress responses and attention allocation could also provide critical information for tackling the high comorbidity rates between mental disorders and ADHD. Nevertheless, many unresolved matters remain. Recent single cell transcriptomic studies identified diverse astrocyte subpopulations throughout the brain [20], highlighting the need to better characterize their roles in attentional processes. It is also important to untangle attention-related subnetworks related to selective, sustained, and divided attention, to consider how each of them is compromised in stress-related disorders, and how the various NVU cell types might contribute to these different domains of cognition.

NVU and cognition in stress-related disorders

Chronic stress is among the main environmental risk factors for developing mood disorders, such as MDD [79]. MDD can adversely impact cognitive function, especially processing speed, attention, and executive function [80,81]. Emerging morphological and functional evidence supports a crucial role for the NVU in mediating the deleterious or adaptive effects of chronic stress in both human and rodent models of stress-related disorders (Figure 2) [3,45–47,82–84]. Thus, NVU pathology may directly contribute to depression-related cognitive impairments.

Stress alters astrocyte regulation of neuronal excitability and synaptic transmission [85]. Regulation of the extracellular content of glutamate at synaptic and extrasynaptic sites is dependent on the activity of its transporters, EAAT1 and EAAT2, in the membrane of astrocyte processes. Reduction in EAAT2 level leads to glutamate uptake disruption and, in rats, it has been shown to cause subsequent excitation–inhibition imbalance and impaired spatial memory (Figure 3A) [86]. EAAT2 loss is found in rodent stress models and postmortem MDD brain tissue (Figures 2C and 3D) (reviewed in [83,87]). Reduction in glutamate astrocytic transporters is not limited to cortical regions but extends to limbic system structures known to contribute to reward processing, behavioral regulation, and mood disorder pathophysiology (Figure 2C) [2,3,83,88]. Genetic deletion or pharmacological inhibition of EAAT2 impact behaviors in rodents [89]. Astrocytes also influence neuronal activity by buffering extracellular potassium following action potentials through $K_{IR}4.1$ channels [90]. Overexpression of astrocytic $K_{IR}4.1$ is sufficient to induce a depressive-like phenotype in rats [91] and increased $K_{IR}4.1$ expression was reported in the parietal cortex of individuals with MDD (Figure 2C) [92]. Moreover, contact between astrocyte end-feet and endothelial cells is highly disrupted in the MDD PFC, with ~50% reduction in colocalization (Figure 2C) [93]. Expression of end-feet aquaporin (AQP)-4 water channels, essential for the control of water homeostasis in the CNS, is also lower in this brain region (Figure 2C) [93]. As for preclinical models, Aqp4-positive astrocyte end-feet are decreased in a genetic rat model with a depression-like phenotype (Figure 1F) [94].

Astrocytes regulate BBB permeability via the release of cytokines, including interleukin (IL)-1β [95], which can be induced by acute stress [96]. In response to IL1β stimulation, astrocytes release vascular endothelial growth factor (VEGF), key for NVU function, promoting greater BBB permeability [95]. VEGF has been implicated in BBB dysfunction leading to the establishment of depressive behaviors [97,98]. Plasma levels of soluble sVEGF165, one of two major protein isoforms of VEGF, is lower in individuals with MDD [97]. Mechanistically, pharmacological inhibition of VEGF receptor 2 with a specific monoclonal antibody prevented chronic restraint stress-induced BBB hyperpermeability and anhedonic behavior in mice [98], highlighting a role for this

vascular growth factor in stress-related disorders. Accumulating evidence suggests that chronic stress can alter BBB integrity contributing to vulnerability versus resilience to stress exposure [45–47,99–101]. For instance, mice susceptible to chronic social stress display a reduction in the endothelial cell tight junction protein Claudin 5 [45–47,99]. However, a subset of mice exposed to the same stressor maintain BBB integrity, Claudin 5 levels, and social interactions. This subpopulation of mice can be seen as resilient to the stressor, with resilience broadly defined as a positive adaptation to adversity [102]. These findings support the notion that individual differences in BBB regulation may contribute to the degree of susceptibility to stress-related disorders. Interestingly, the forementioned reduction of Claudin 5 under stress is sex specific, with a reduction in males found in the nucleus accumbens (a key hub of the reward circuit) [45,99], whereas Claudin 5 is reduced in the PFC in females [46] (Figures 2A and 3B). Targeted disruption of the BBB with viral downregulation of Claudin 5 in these brain regions was sufficient to promote depression-like behaviors [45,46], reinforcing a central role for the neurovasculature in stress-related disorders in line with clinical evidence of BBB disruption in human MDD and other psychiatric illnesses [45–47,83,84,93,103–105]. Comparative transcriptomic analysis of the endothelium in vulnerable brain regions from stress-susceptible versus -resilient male and female mice revealed sex-specific gene expression patterns [46,47] as well as a permissive transcriptional state of histones surrounding the *CLDN5* gene promoter [47]. Deciphering how molecular and cellular processes modulate the BBB and NVU properties in the context of acute and chronic stress could help design novel therapeutic strategies to treat mood disorders and alleviate depressive symptoms, including those associated with neurodegenerative diseases and dementias.

Toolboxes and novel approaches to study the NVU

Molecular profiling of NVU-related cells and new-generation sequencing techniques

To facilitate the identification of relevant targets in NVU cells, it is ideal to establish cell-specific transcriptional and translational profiles. Most neurovascular cells are relatively easy to purify by immunopanning, fluorescence- or magnetic-activated cell sorting using specific markers [46,47,106,107]. They can also be isolated with reporter mouse lines under specific promoters or with transgenic mouse lines that express GFP-tagged ribosomes for immunoprecipitation (astrocytes: *GFAP*, *Aldh1l1*, *Glt1*, and *S100b*; endothelial cells: *Tie-2*, *VE-Cad*, etc.) (Figure 4A) [108].

Transcriptional profiling of any purified cell type can be unraveled through sequencing techniques, such as RNA-seq (Figure 4E). With this method, transcript abundance is directly proportional to the number of sequences reads, and it allows exploration of alternative splicing, a key factor underlying transcriptome diversity [109]. Since early transcriptomic analyses of purified NVU cells from the mouse cortex [109], several databases and studies have been published in both mouse and human tissues under different conditions (Tables 1 and 2) [106,110–114]. RNA-seq is decreasing in cost and increasing in accessibility for researchers, with various protocols now available. Despite major advances, RNA-seq is not without limitations. The averaging that occurs in pooling large numbers of cells does not allow for detailed assessment of the fundamental biological unit (the cell) and the intrinsic cellular diversity within populations. Nevertheless, this technique remains a staple in neuroscience research and has led to important findings, such as identification of transcriptional profiles associated with human cognition [115] and MDD (Figure 4E) [116].

The first single-cell (sc)RNA-seq study, published in 2009, aimed to assess previously unappreciated levels of heterogeneity within cell populations [117]. scRNA-seq also provides important information, including monoallelic gene expression [118], splicing [119], and gene co-expression patterns [120]. Within neurovascular populations, scRNA-seq revealed a remarkable diversity of vascular cell transcriptomes [10,121,122]. Region-specific heterogeneity within

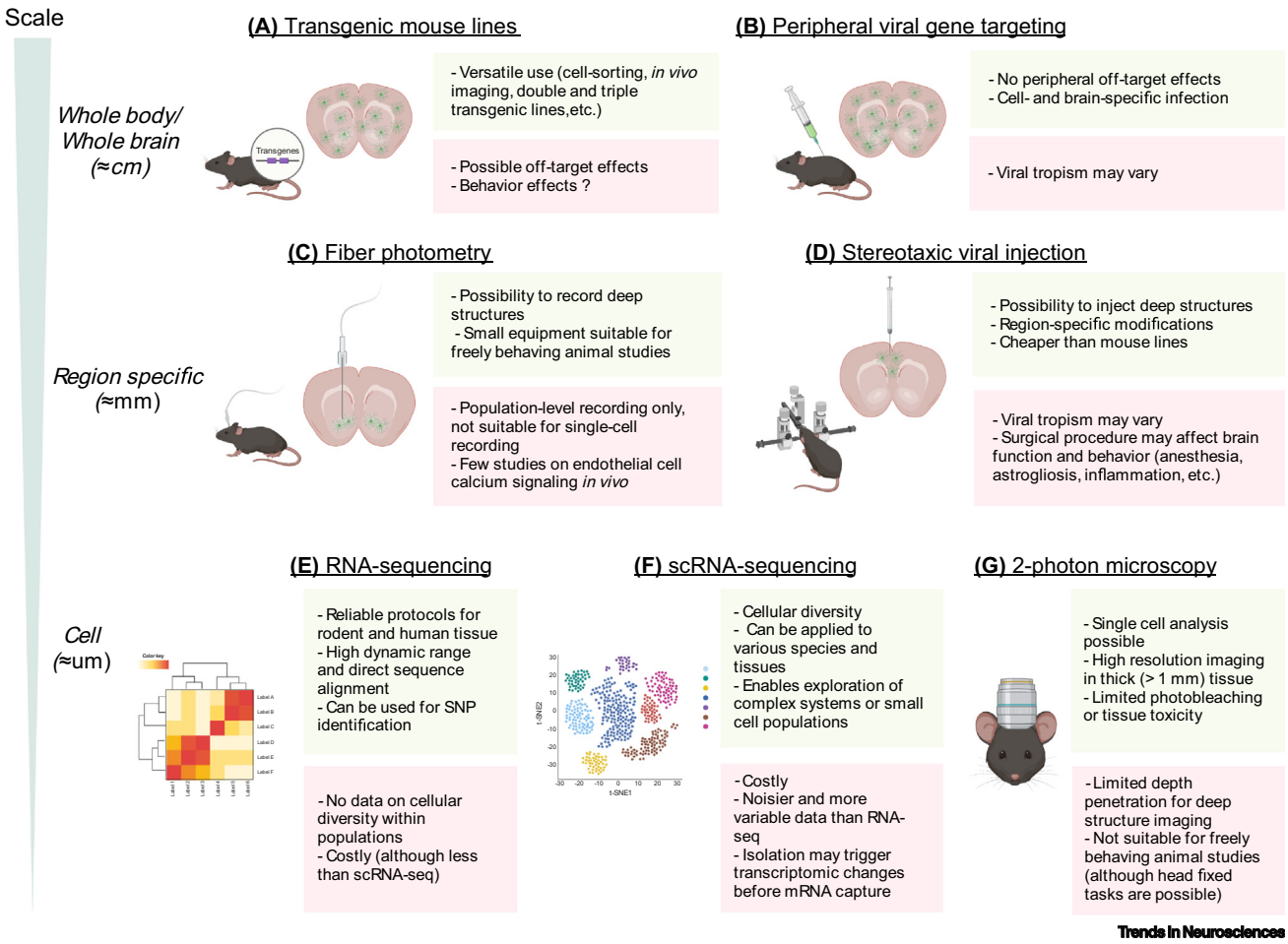

Figure 4. Tools to study neurovascular unit (NVU) functions and properties in health and diseases. For each method, green squares summarize the advantages and red squares list some of the pitfalls and shortcomings. Transgenic mouse lines can be used for whole-body transgene expression or deletion (A) [108]. For brain-specific modulation, several viral serotypes have been developed with high tropism for specific neurovascular cells (B) [133,148,149,151]. Brain-region specific recordings of calcium transients can be achieved through fiber photometry (C) [130,138,139], while functional manipulations can be performed with stereotaxic viral injections (D) [45,46,150]. At the cellular level, RNA-sequencing (RNA-seq) can inform on population-level transcriptomic changes (E) [106,109–114]. Single cell (sc)RNA-seq allows for probing diversity within cell populations (F) [20,117,121,122]. For *in vivo* investigation of cell populations, two-photon microscopy grants high-resolution access to the brain with limited photobleaching or tissue toxicity (G) [141–144]. Figure created using BioRender (www.BioRender.com).

astrocyte subtypes was also reported [20]. NVU cell heterogeneity may contribute to the regional alterations observed in cognitive- and stress-related disorders (Table 2 and Figure 4F).

One considerable barrier to scRNA-seq is the effective isolation of single cells from the tissue of interest. Some cell types may be bound to extracellular structures or neighboring cells or, alternatively, might lack specific markers (e.g., pericytes), resulting in sample contamination. Additionally, scRNA-seq operates under the assumption that isolation of single cells from their microenvironment does not trigger rapid artifactual transcriptomic changes before mRNA capture [123]. Alternate strategies, such as single nuclei RNA-seq (snRNA-seq) [124], fixation to stabilize and preserve cells without compromising RNA integrity [125,126], or controlling for temperature and providing inhibitors to reduce shifts in cellular transcriptional state during enzymatic digestion steps, could help to overcome microenvironment-related limitations [127]. An innovative approach is Live-seq, a single-cell transcriptome-profiling technique preserving cell viability and

Table 1. RNA-seq and single cell RNA-seq databases related to neurovascular cells

Cell type	Brain region and/or organ	Species	Refs
Endothelial cells	Whole brain (also liver, kidney, testis, small intestine, colon, spleen, skeletal muscle, heart)	Adult male mice	[110]
	12 organs: adipose tissue, aorta, brain, diaphragm, heart, kidney, liver, lung, mammary gland, pancreas, skeletal muscle, trachea	Pooled male and female adult mice	[121]
All cell types	Frontal cortex, striatum, thalamus, hippocampus, posterior cortex, cerebellum, substantia nigra and ventral tegmental area, globus pallidus externus and nucleus basalis, entopeduncular nucleus and subthalamic nucleus	Adult male mice	[111]
Mural cells (*Pdgfrb*-GFP; *Cspg4*-DsRed); endothelial cells (*Cldn5*-GFP;*Cspg4*-DsRed); perivascular cells (*Pdgfra*-H2BGFP); smooth muscle cells (*SM22-Cre; R26-stop-tdTomato*)	Whole brain, lung	Adult male and female mice	[106]

stability during RNA extraction using fluidic force microscopy. It withdraws a small, high-quality amount of cytoplasmic mRNA from live single cells to perform transcriptional profiling. Interestingly, Live-seq can directly couple the current state of a cell to its downstream molecular and phenotypic properties and can be used to perform longitudinal sequential molecular profiling of the same cell [128]. Pitfalls of basic scRNA-seq can also be overcome by spatial transcriptomics, an innovative approach allowing observation of cells in intact tissue, which provides valuable

Table 2. RNA-seq and single cell (sc)RNA-seq databases related to neurovascular cells in disease models or aging[a]

Technique	Condition	Cell type	Brain region and/or organ	Species	Refs
RNA-seq	Seizure (kainic acid), stroke (permanent MCAO), EAE, TBI	Endothelial cells (Rosa-tdTomato; *VE-Cadherin*-CreERT2)	Seizure: forebrain MCAO: ipsilateral region EAE: spinal cord TBI: ipsilateral cortical lesioned region	Adult male mice[b] (MCAO, TBI, seizure); adult female mice[b] (EAE)	[112]
RNA-seq	Healthy brain, epilepsy, cancer	Astrocytes	Temporal lobe cortex, gray matter from whole cortex	Fetal human brain tissue; human (juvenile and adult); mouse (1-, 4-, 7-, and 9-month old)	[113]
RNA-seq	MDD and chronic stress (mice)	Bulk brain tissue	Ventromedial PFC, orbitofrontal cortex, dorsolateral PFC, anterior insula, nucleus accumbens, ventral subiculum	Human (men and women); mouse (male and female)	[116]
Clariom S assay[c]	Chronic stress	Endothelial cells	Nucleus accumbens	Adult male mice	[47]
			Medial PFC	Adult female and male mice	[46]
scRNA-seq	Aging	Endothelial cells, microglia, pericytes, smooth muscle cells, astrocytes, oligodendrocytes, neurons	Whole brain	Young (3-month old) versus aging (28-month old) male mouse brain	[122]
VINE-seq[d]	AD	Brain microvessels	Hippocampus and cortex	Human	[114]

[a]Abbreviations: EAE, experimental autoimmune encephalomyelitis; MCAO, middle cerebral artery occlusion.
[b]Adult mice (2–3-months old) were used in each disease model except for TBI (PND 21).
[c]Clariom S assay: transcriptome-wide gene expression profiling of >20 000 well-annotated genes (only exons).
[d]VINE-seq: vessel isolation and nuclei extraction for sequencing.

information about the position of cells as well as their cellular state, phenotype, and, ultimately, tissue function [129].

New-generation sequencing techniques offer unprecedented access to cellular identity, states, and heterogeneity, which can be explored in various brain regions and under different conditions to investigate mechanisms of cognitive- and stress-related disorders with high translational potential from rodents to humans in both sexes. However, restricted temporal resolution of these methods needs to be carefully considered when drawing conclusions, given that generating longitudinal data within animals are not yet possible. Thus, *in vivo* approaches are still necessary to describe complex and evolving cellular states underlying behavior.

In vivo imaging and functional measurement of calcium signaling

Unlike neurons, astrocytes, endothelial cells, and mural cells are not electrically excitable. However, they can be monitored by measuring Ca^{2+} signaling. Spatiotemporal calcium elevations resulting from cellular activation can further affect downstream processes. In astrocytes, Ca^{2+} elevations precede the release of gliotransmitters modulating synaptic transmission. Endothelial cell calcium dynamics regulate physiological processes, such as cell migration and angiogenesis [130] and, similarly, VSMC Ca^{2+} elevations inform the contractile state of the barrier through vasoconstriction and vasodilatation processes [131]. Astrocytic Ca^{2+} signaling stems from different sources, including pumps, organelles, receptors, and channels. The relative contribution of each of these sources to overall Ca^{2+} dynamics is not always clear and could vary across brain regions and cell types [132,133]. The most widely used genetically encoded calcium indicators for studying real-time Ca^{2+} dynamics *in vivo* are members of the GCaMP family, which can be introduced in a cell type-specific manner using viral vectors containing the Cre-loxP system (Figure 4) [134]. For astrocyte manipulation in adult mice, the tetracycline-inducible Aldh1l1-Cre/ERT2 transgenic line is arguably the best option currently, offering temporal control of gene expression, high cell specificity, and efficiency [134,135]. For endothelial cells, mice constitutively expressing GCaMP2 under the connexin 40 promoter have been among the most widely used methods. Unfortunately, low dynamic range and high baseline fluorescence limit the practicality of this approach, as well as its inability to image endothelial Ca^{2+} signals in veins [136]. A newly developed mouse line constitutively expressing GCaMP8 under the endothelium-specific VE-cadherin promoter largely resolved these issues [136], rendering it a useful tool to study complex calcium dynamics in endothelial cells. Finally, Ca^{2+} dynamics in VSMCs can be investigated with acta2-GCaMP-GR transgenic mice or, more broadly in VSMCs and pericytes, with Pdgfrb-CreERT2:R26-GCaMP6s$^{f/stop/f}$ mice [131,137]. To our knowledge, these lines have not yet been used in the context of stress and cognition research.

To better understand how Ca^{2+} transients are behaviorally relevant and their implications in cognitive function, it is necessary to study Ca^{2+} dynamics *in vivo* in freely behaving animals. Fiber photometry is a relatively inexpensive and reliable approach enabling cell type-specific longitudinal analysis of cells (Figure 4C). Due to their small size, fiber cannulas are more suitable for freely behaving animal studies compared with cameras or lenses, and can be implanted in multiple brain areas simultaneously [138], even within deep regions, such as the striatum [139]. However, resolution of fiber photometry is limited to population-level imaging (i.e., the cells neighboring the cannula) and the method cannot record individual cells. To our knowledge, no fiber photometry studies in endothelial cells have been conducted to date. Little is known about how calcium signaling is controlled and coordinated within endothelial cells and across vascular beds. The lack of quantitative approaches to comprehensively characterize live endothelial Ca^{2+} activity *in vivo* remains a challenge [130].

Two-photon imaging can be used to overcome the lack of single cell resolution in fiber photometry, offering a visual representation of cellular dynamics and morphology in live tissue (Figure 4G).

It is superior to confocal microscopy because it excels at high-resolution imaging in intact thick tissue (up to ~1 mm) with limited photobleaching or phototoxicity due to reduced laser power and restricted scanning of the focal plane [140]. However, limited penetration depth does not allow imaging of deep structures [141] and the size of the imaging apparatus restricts the potential for freely behaving animal studies, although mice can be trained to perform head-fixed behavioral tasks [142,143]. Nevertheless, this technology is extremely versatile and can be exploited to study neurovascular coupling and CBF [144] and to perform single cell optical ablations [145]. Finally, brain miniscopes [146] or mini-endoscopes [147], originally developed to visualize neuronal activity, can be used to study the NVU in freely behaving animals, although neurovascular-related publications taking advantage of these innovative tools remain sparse.

Viral manipulations

A limitation of traditional transgenic mouse lines is that they express the desired transgene constitutively, which could affect peripheral organs, functions, and behavioral outcomes. Gene delivery through viral vectors is an alternative approach to modulate brain cells in a region- and cell type-specific manner with minimal off-target effects. Several lentivirus and adeno-associated virus (AAV) serotypes have been described that target neurovascular cells with high specificity [133,148]. Some serotypes display natural selectivity for astrocytes or endothelial cells, but specificity can also be enhanced by combining a cell-specific promoter or enhancer to the construct [133,148,149]. Region-specific downregulation of Claudin 5 expression with an AAV2/9 short hairpin (sh)RNA alters emotion-related behaviors [45,46]. However, targeted suppression of the same tight junction protein decreases cerebral edema, improving cognitive outcome following TBI [150] (Figure 4D). These findings highlight the complex role of the BBB and NVU in cognitive and emotional processes and the benefits of using viral approaches to target specific brain regions in various contexts.

Beyond region-specific alterations of gene targets using stereotaxic surgeries, several AAV variants with high specificity for neurovascular cells through peripheral injections have been described (Figure 4B). For example, intravenous administration of AAV-PHP.eB coupled with the *GfaABC1D* promoter enables high and efficient transduction of astrocytes following a single intravenous injection [133,151]. Similarly, AAV-PHP.V1 has exceptionally high tropism for brain endothelial cells following a single intravenous injection [151]. These viral serotypes with high affinity and transduction potential for NVU cells could have significant impacts for future studies, because they are cheaper than generating transgenic mouse lines and restrict the potential deleterious peripheral effects of whole-body transgene modifications. Importantly, they are also less damaging for brain tissue compared with stereotaxic surgeries when region specificity is not needed. However, viral tropism depends on several factors, including brain region, mouse background, and administration route, and appropriate controls should be added when designing experiments [133].

Altogether, these innovative techniques will contribute to a better understanding of how NVU cell types affect cognition and behavior in both health and disease. As outlined here, interesting connections can be drawn between NVU function and behavior, but more work is needed to unravel the precise mechanisms, a necessary step to develop novel cognition-modulating therapies. We underscore in this context the need for selective and precise targeting of each NVU cell type, although admittedly not all components of the NVU are equally accessible experimentally and will require new tools and approaches, including those discussed above. Combining transcriptional, morphological, functional, and behavioral assays is a powerful approach, considering that each method provides different advantages and limitations (Figure 4), which should be carefully assessed when designing experiments.

Concluding remarks

Classically, animal and human behavior was thought to be determined by neuronal activity alone; however, increasing evidence highlights a role for several NVU-related cell types in modulating brain function and behavior. Astrocytes are perfectly positioned to bridge neuronal and vascular activity, but endothelial and mural cells are also emerging as protagonists in shaping and modulating behavior, among their other crucial functions. Despite preclinical and clinical findings supporting neurovascular alterations in psychiatric illnesses, such as MDD, bipolar disorder, or schizophrenia, underlying molecular and cellular mechanisms through which changes in the NVU affect cognition, mood, and stress responses remain elusive.

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Declaration of interests

The authors declare no financial or competing interests.

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Outstanding questions

Altered astrocytic glutamate transport in the hippocampus is linked to impaired spatial memory. Conversely, upregulation of astrocytic EAAT2 in the dorsal striatum is associated with loss of behavioral flexibility. This suggests heterogeneity in the distribution and function of NVU cells as an important factor contributing to region-specific effects of altered NVU function. Could this diversity also have an impact on cognitive processing under stress conditions?

Astrocyte-mediated signaling is central to neurovascular coupling and recent evidence suggests that astrocyte dysfunction underlies abnormal resting-stage functional connectivity in depression. Could disruption of astrocyte-mediated signaling drive the cognitive deficits associated with stress-related disorders and other psychiatric illnesses?

Cerebrovascular alterations can lead to cognitive impairments, but can they originate from improper neuronal regulation affecting NVU function? Is promoting neurovascular health with interventional strategies, such as diet or physical exercise, sufficient to protect or restore cognitive function, and how do such interventions affect NVU cells?

Sex differences are well documented in the prevalence and comorbidities of stress-related disorders. How does sex contribute to NVU regulation and what mechanisms underlie the sex-specific cognitive patterns observed in mood and neurodegenerative disorders?

Inflammation, a hallmark of neurovascular dysfunction and stress-related disorders, is also observed in the most common causes of dementia, namely AD, stroke, vascular cognitive impairment, Parkinson's disease, frontotemporal dementia, and amyotrophic lateral sclerosis. Could inflammation-driven vascular dysfunction potentiate cognitive impairments in these disorders? Could anti-inflammatory treatments dampen the progression of cognitive impairments?

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