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Polypharmacology approaches for brain disorders aimed to enhance brain permeability and circadian clock targeting☆

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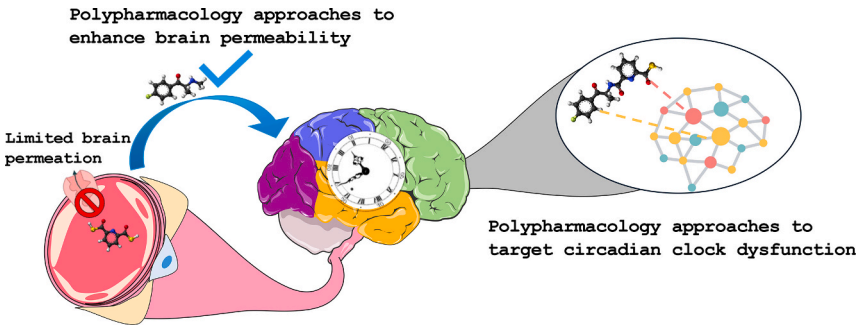
HIGHLIGHTS

- Circadian rhythm disruption (CRD) is associated with several brain disorders.
- Polypharmacology offers strategies to improve CNS drug delivery and modulate complex molecular networks in CRD.
- Drug combinations, co-drugs and targeted prodrugs may enhance CNS drug delivery.
- Drug combinations and MTDLs can modulate multiple targets of the CRD network, offering potential CNS disorder treatments.

GRAPHICAL ABSTRACT



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ABSTRACT

Circadian rhythm disruption (CRD) is a common feature of several brain disorders. The restoration of circadian clock function and the development of circadian-based therapies may have significant therapeutic implications for brain diseases that extend beyond sleep disorders. However, several challenges persist due to the complexity of circadian interactions with multiple cellular pathways underlying CRD in brain diseases, together with the CNS compartmentalization, including the presence of the blood–brain barrier (BBB). Against these drawbacks, polypharmacology is a promising strategy to potentially provide greater efficacy by targeting multiple components of the CRD network through drug combinations or multi-target-directed ligands. Polypharmacology also offers innovative approaches to brain drug delivery by enhancing BBB penetration of CNS-directed drugs using combinations, co-drugs, and targeted prodrugs. Herein, we review polypharmacological strategies to improve BBB permeability of CNS agents and suggest the exploitation of polypharmacology as a promising new avenue for circadian clock modulation in the treatment of brain disorders.

1. Introduction

The evolution of living organisms is based on their ability to adapt to environmental changes. The perpetual and periodic 24-h rotation of the Earth on its axis has led organisms from all phyla to develop internal "biological clocks" in order to anticipate recurring daily alterations in

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their external environment. These clocks, termed "circadian clocks" (CC) from the Latin *circa* (about) and *diem* (day), govern nearly all physiological processes with a period of about 24-h, inducing rhythmic changes in gene and hormone expression, and regulating functions such as body temperature, renal or cognitive functions, and metabolism [1]. This evolutionary preserved homeostatic mechanism is of paramount importance in maintaining health, as evidenced by the fact that circadian rhythm disruption (CRD) is a common denominator of cancer and a variety of neurodegenerative, metabolic, and sleep-related disorders [2]. It is well accepted that several factors, including genetic and environmental, along with hormonal imbalances, contribute to CRD and lead to diseases with different etiology and complexity. However, elucidating the complex relationships between circadian disruption and diseases, other than sleep disorders, is increasingly challenging [3]. It is possible that CRD is not only a cause, but also a consequence or determinant of such diseases. Nevertheless, the restoration of CC and the development of appropriate therapeutic interventions may have significant implications for human health that extend beyond sleep disorders. As such, the development of circadian-based treatments is a critical test of the hypothesis that CRD is a key therapeutic point of intervention.

In addition to insomnia and other sleep disorders [4], there has been a significant drive over the past decade to develop CC modulators for the treatment of several other central nervous system (CNS) diseases [5]. Among these compounds, just a few have optimal brain bioavailability and pharmacodynamic properties, enabling pre-clinical in vivo animal studies [5]. Indeed, the complex interactions between circadian disruption and the multiple diverse cellular pathways involved [6,7] along with the CNS compartmentalization, are some of the main challenges on the road to therapeutics.

Polypharmacology approaches appear particularly promising to face such intrinsic complexity. Polypharmacology, from the Greek prefix *poly* (many) and the words *pharmakon* (drug) and *logos* (study), refers to the simultaneous modulation of two or more targets involved in the complex disease network by drug combinations or multi-target-directed ligands (MTDLs) [8,9]. As such, these may result in a better modulation of the CRD network and consequently in an improved clinical efficacy and safety profile [8,9]. In addition, the CNS compartmentalization and the existence of the blood–brain barrier (BBB) are critical obstacles in the development of novel CC-targeting compounds since they can limit the influx of circulating molecules into the brain [10]. Polypharmacology may offer several potential advantages also when applied to brain drug delivery. Polypharmacology-based strategies may involve designing drugs with specific physicochemical properties that enhance BBB penetration, such as increased lipophilicity. Other effective polypharmacological tools include, among others, the design of targeted prodrugs, co-drugs, or the co-administration of efflux pump inhibitors. Brain drug delivery can also be achieved by using nanoparticle carriers that can cross the barrier more effectively, which will not be the topic of the review. Additionally, combination therapies have been explored aimed at exploiting the BBB as a *carrier*, rather than a *barrier* to efficiently drive therapeutics into the brain.

Herein, we provide an overview of healthy and diseased circadian systems, highlighting the extensive crosstalk between circadian rhythms, and multiple pathways involved in CNS diseases. We also describe polypharmacology approaches and BBB structure, function, and transport mechanisms, while reflecting on how polypharmacology can be a valuable tool to enter the brain and modulate CRD.

2. The circadian clock: Physiology and dysregulation

2.1. Overview of the mammalian circadian clock

Circadian clocks (CC) are ubiquitous and evolutionarily conserved across all organisms; they are *entrained* (synchronized) to the 24-h day-night cycle, through environmental stimuli called *zeitgebers* (German for time-givers). The most potent zeitgeber is light, although it has been demonstrated that temperature, exercise, medications, and food intake play important roles [1,11,12] **(**Fig. 1**)**. In 1729, the French researcher Jean-Jacques de Mairan laid the foundations of modern chronobiology by demonstrating that the leaves of the mimosa plant periodically open and close during the day, even in the absence of light. Since this behavior was not a direct response to external conditions, it suggested the existence of an intrinsic biological clock. By conducting sleep experiments in conditions of constant darkness, researchers were able to replicate de Mairan's findings in humans, showcasing regular diurnal sleep-wake patterns and temperature fluctuations, irrespective of external stimuli [1].

Although initially believed to exist only in the brain, mammalian circadian clocks are present in all tissues and exhibit bottom-up and top-down organization. Bottom-up organization involves individual cells of the periphery that can generate daily rhythmic patterns and form networks that regulate the function of tissues and organs. On the other hand, top-down organization is governed by the master clock residing in the suprachiasmatic nucleus (SCN) of the hypothalamus. Light stimuli from intrinsically photosensitive melanopsin-expressing retinal ganglion cells activate the SCN, which subsequently orchestrates the behavior of other brain areas or peripheral tissues, regulating the production of neurotransmitters and hormones [2]. Specifically, SCN-driven cortisol and melatonin rhythms synchronize central and peripheral clocks, while insulin pulses entrain metabolic oscillators to feeding times. Chronic misalignment of hormonal rhythmicity—through shift work, nocturnal light exposure, or irregular feeding patterns—disrupts metabolic homeostasis and elevates the risk of insulin resistance, obesity, type 2 diabetes, and cognitive defects [13].

On the molecular level, clock elements are engaged in a constant rhythmic transcriptional and translational feedback loop (TTFL). This mechanism, whose discovery was worth the Nobel Prize in 2017, is evolutionarily conserved across the animal and plant kingdoms and has a period of almost 24-h. The function of the TTFL is governed by interactions between activators and repressors (Fig. 2**)**. Briefly, circadian locomotor output cycle kaput (CLOCK) and brain and muscle aryl hydrocarbon receptor nuclear translocator like 1 (BMAL1) heterodimerize upon activation of the loop (daytime) and bind to E-box enhancer elements of a plethora of genes. Notably, they drive the expression of repressors period (PER) 1–3 and cryptochrome (CRY) 1–2. At night, PER and CRY also heterodimerize and interact with other core components of the clock, such as casein kinase 1-delta (CK1δ) and CK1ε, ultimately suppressing BMAL1 and CLOCK transcriptional activity. Finally, ubiquitination of PER and CRY proteins leads to their proteasomal degradation, alleviating their suppressor effects and leading to the start of a new 24-h cycle. Although this is the central loop of the circadian system, secondary TTFLs such as the ROR/REV-ERB loop coordinate and stabilize its function through the modulation of *Bmal1* gene expression [14,15] **(**Fig. 2**)**.

2.2. Circadian dysfunction and its role in disease

Various aspects of the modern world, such as nighttime illumination and the blue light emitted from screens, shift work, and the regular use of alarm clocks, as well as erratic feeding patterns, can have profound health effects. The resulting CRD, i.e., the misalignment of the internal CC, is widely overlooked but can lead to severe homeostatic imbalance. Robust evidence from epidemiological studies suggests a strong correlation between long-term shift work and the development of cancer and metabolic/gastrointestinal disorders. Even more concerning is the fact that almost 70 % of the population faces social jet lag, which is defined as the difference between median sleep times on workdays and free days. This phenomenon, deeply integrated into the modern lifestyle, is linked to the development of type 2 diabetes, addiction to caffeine, nicotine, and alcohol, as well as cardiovascular and neurodegenerative diseases [16,17] **(**Fig. 3**)**.

The importance of maintaining circadian homeostasis can be

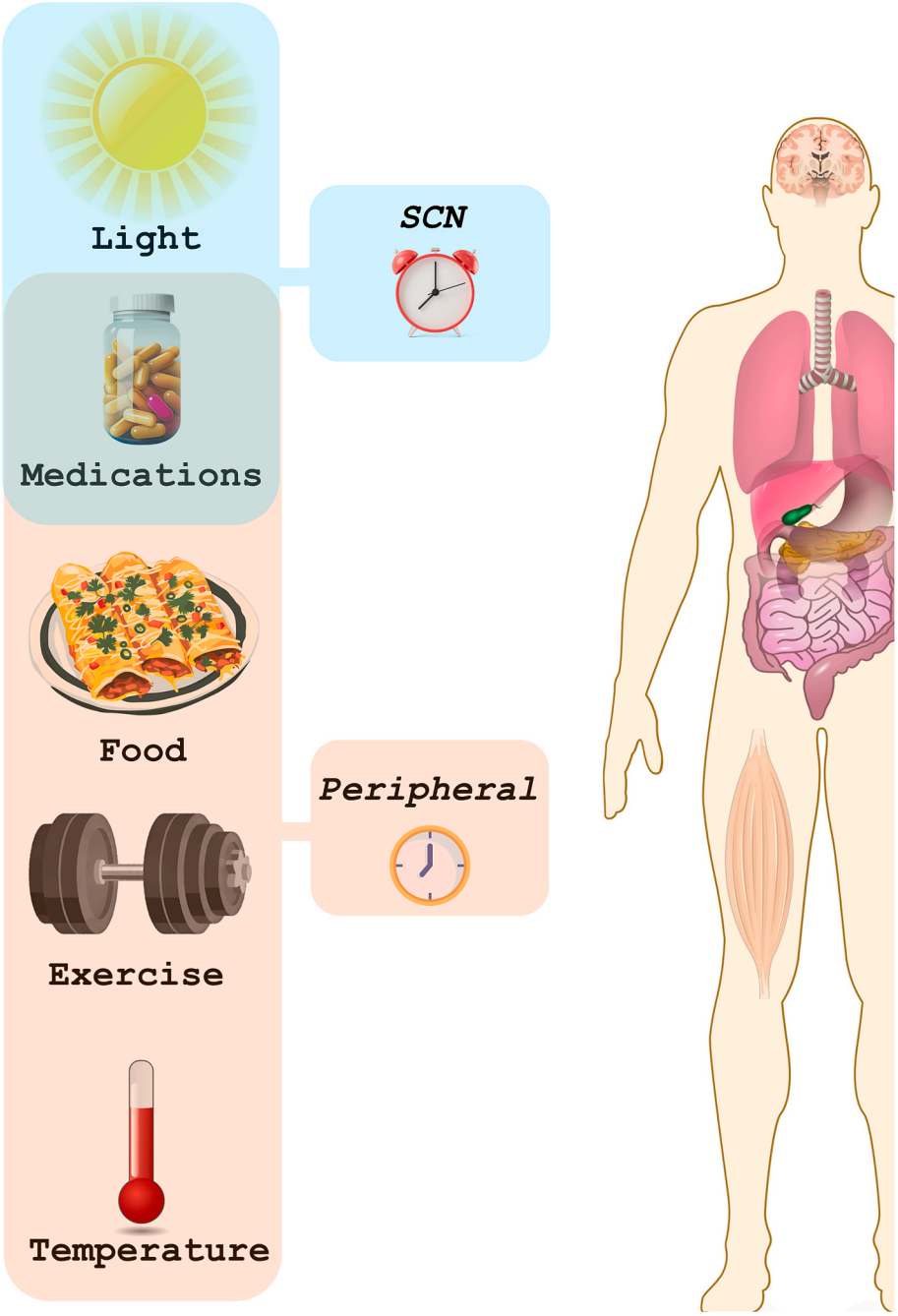

Fig. 1. Main zeitgebers affecting the central and peripheral clocks.

demonstrated by the fact that CRD is involved in the pathogenesis of a wide range of disorders, such as cancer and brain disorders [5]. Acute phenomena such as myocardial infarctions are also linked to the circadian clock since the majority of cases occur during morning hours. Moreover, experimental mice models lacking a functional CC display metabolic defects and shorter lifespans than wild-type mice [18], while in humans, familial advanced sleep phase syndrome stems from genetic mutations in key clock components [19].

Circadian misalignment and brain disorders

The interplay between the CC and CNS disorders is vast and complex. Firstly, in neurodegenerative diseases, it is well demonstrated that aging is one of the major associated risk factors. One predominant feature of aging neurons is the dampening of their CC, as SCN neurons begin losing phase coherence and vasopressin-expressing cells. The magnitude and precision of gene expression management and synchronization of neuronal activity decline in the SCN, while peripheral clocks also suffer from functional deficits [7]. In the aging brain, secretion of hormones such as melatonin and cortisol appears to be dysregulated, while sleep patterns become more erratic owing to CRD [20]. Notably, more than 80 % of people with REM sleep behavior disorder will develop Parkinson's disease (PD) or dementia [7]. In Alzheimer's disease (AD), one of the earliest biomarkers is sleep impairment, which precedes cognitive decline. Deposition of β-amyloid (Aβ) exhibits diurnal oscillations, with levels being higher in the active phase and lower while resting. On the other hand, Aβ clearance via the glymphatic flow happens mainly during sleep. This suggests a possible link between the sleep-wake cycle and AD pathogenesis. Importantly, while sleep disturbances may drive amyloid plaque formation, Aβ plaque burden can result in sleep fragmentation, creating a vicious cycle that promotes AD progression [21].

Single polymorphisms in the genes that encode BMAL1 and PER1

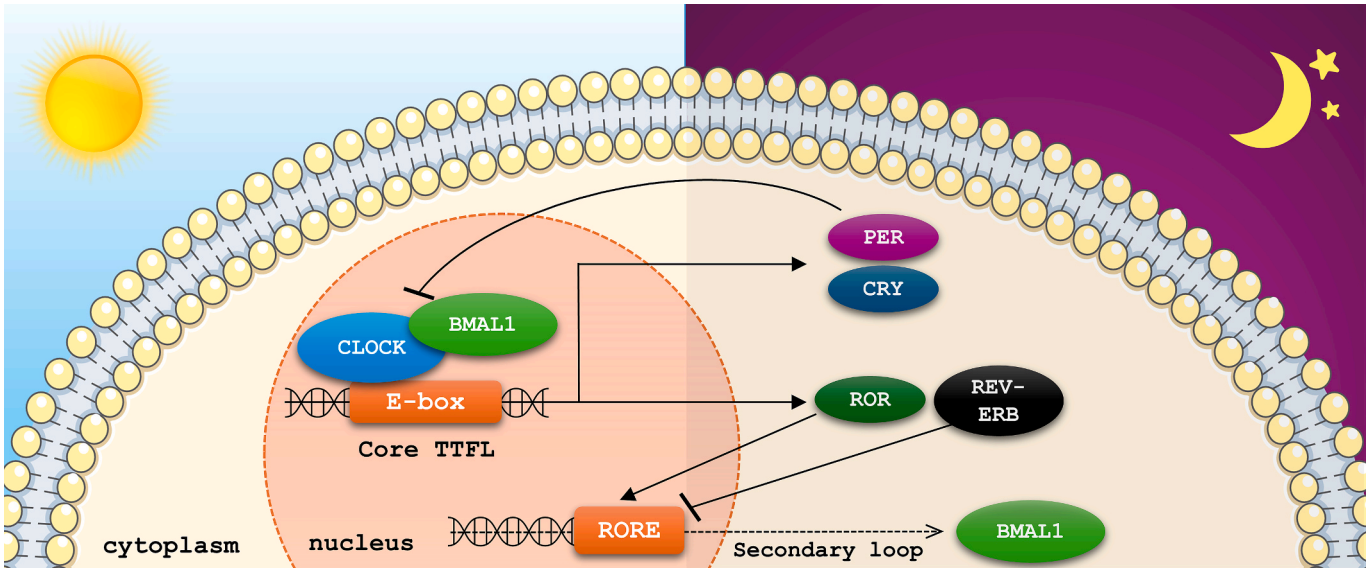

Fig. 2. Molecular mechanisms of CC function.

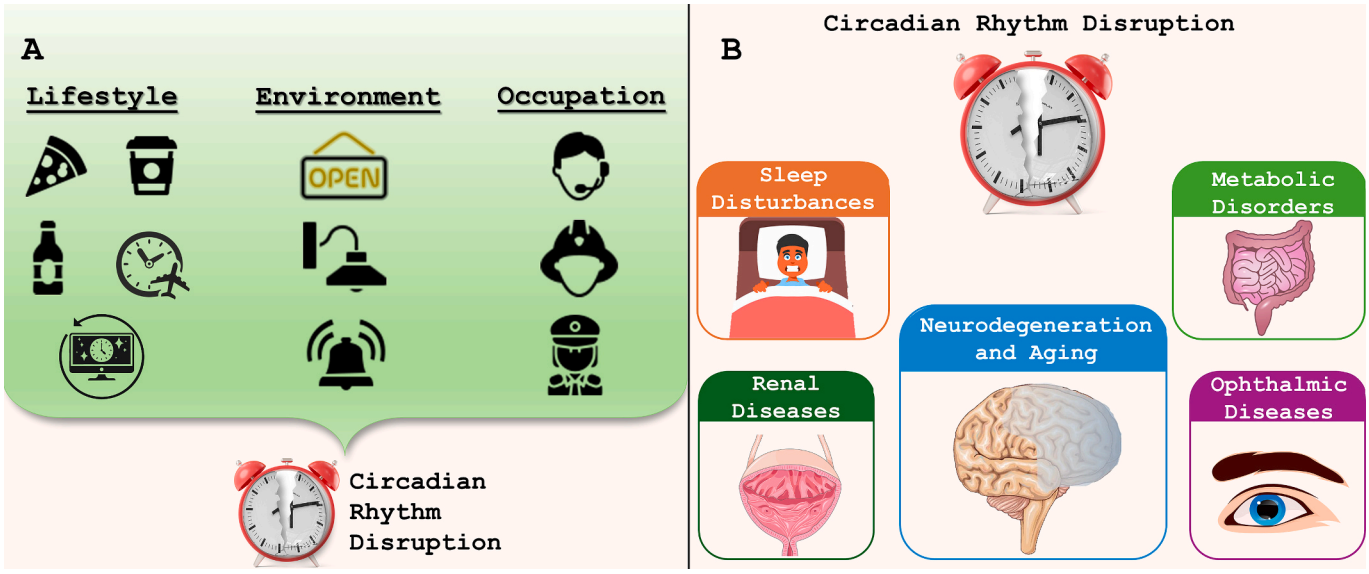

Fig. 3. (A) Main factors disrupting normal circadian rhythms; (B) the vital role of CRD in the pathogenesis of diseases.

have been correlated with the development of PD, while the expression of presenilin-2, a regulator of Aβ levels, is controlled by CC [22]. Moreover, the production and elimination of reactive oxygen species (ROS) adhere to rhythmic diurnal patterns. While awake, increased neuronal activity results in higher levels of ROS, which are then eradicated by ROS scavengers and antioxidants during the resting period. Disruption of the circadian network can result in excessive ROS burden, as demonstrated by studies in BMAL1 deficient mice [7,22]. Since most of the metabolic processes are governed by the CC, and ROS are formed as metabolic by-products, there is a clear possibility that CRD could lead to oxidative stress [20]. Moreover, several lines of evidence associate CRD with a higher prevalence of mental disorders such as major depressive disorder or anxiety [23]. Studies in humans and rodents have provided a clear link between aberrant circadian rhythms and the pathophysiology of depression. Although a definite mechanism has not yet been elucidated, CRD has been associated with depressive symptoms through a disruption of neurogenesis and a decrease in melatonin levels in the brain [24]. Finally, circadian dysfunction negatively affects the BBB integrity, further contributing to the development of neurological disorders, as will be discussed in **Section 5.1**. A growing body of evidence indicates a complex and intricate relationship between CRD and glioblastoma progression. Disruptions in circadian rhythms have been shown to influence several key processes in glioblastoma, including tumor growth, invasion, and response to treatment [25].

Although the link between CRD and the development of brain disorders is robust, further research is needed to elucidate their relationship at the molecular level. Is the disruption of circadian function a predominant cause of disease, or merely a result of neuronal abnormalities? Can this interplay reveal new avenues towards the treatment of CNS disease and reignite the hope for life-saving medication?

3. Polypharmacology: Overview and current perspectives

Polypharmacology is defined as “the design or use of pharmacological agents that act on multiple targets or disease pathways” in the thesaurus of the National Library of Medicine [26]. Hence, it entails the

rational design of single compounds acting on multiple therapeutic targets (termed "multi-target-directed ligands, MTDLs" by our group [9]), as well as the combination of different active pharmaceutical ingredients (APIs) in the same dosage form (fixed-dose combination), or in distinct ones administered simultaneously **(**Fig. 4**)** [27]. More than 40 years ago, Philip Salvatore Portoghese proposed the development of bivalent ligands for opioid receptors possessing greater narcotic antagonistic potency than their monovalent congeners [28].

Therefore, the concept of single drugs possessing selective promiscuity is not a novel idea. However, skepticism from academia and industry alike sidetracked this notion for decades, until groundbreaking work from Morphy and others established MTDLs as a valid, promising therapeutic strategy [9,29,30]. The complex etiology of multifactorial diseases has provided fertile soil for polypharmacology. According to the perspective of network medicine and system biology [31], these diseases arise from the breakdown of regulatory molecular networks, which may explain the profound inefficacy of current single-target treatments. Polypharmacology opts for the additive or synergistic modulation of multiple interconnected targets [32,33], providing a holistic approach to a convoluted problem, including brain disorders and CRD. In principle, both combinations and MTDLs are equally feasible for achieving the desired additive or synergistic effects. However, limited patient compliance and the risk of drug-drug interactions (DDIs) pose significant challenges in the development of combination regimens **(**Fig. 4**)** [34]. Furthermore, aging, comorbidities, and subsequent polytherapy significantly contribute to increasing the risk of pharmacological side effects (e.g., liver injury) and drug interactions in many AD patients [34]. Importantly, appropriate combination dosing of two APIs to afford suitable concentrations of both drugs at the site of action (e.g., the brain) can be difficult because of their different physicochemical and pharmacokinetic properties. Combination therapies can also include one or more drugs with one or more other types of treatment modalities, such as devices, light, or lifestyle interventions [35]. Regardless of the individual agents involved, all combinations that produce additive or synergistic effects are referred to as pharmacodynamic combinations. There are also pharmacokinetic combinations, which include one or more APIs intended to prevent or slow the metabolism of the active agent, antagonize peripheral side effects, or facilitate the access of the API to the CNS. These have demonstrated great clinical utility in affecting a drug's absorption, distribution, metabolism, or elimination (ADME) properties [35]. Examples of pharmacokinetic combinations include L-dopa plus a dopamine decarboxylase inhibitor for the treatment of PD. MTDLs are instead single-molecule polypharmacology tools modulating multiple targets. Inherent advantages of MTLDs compared to combinations relate to the improved patient compliance and the absence of DDIs **(**Fig. 4**)**. In addition, the prediction of pharmacodynamic/pharmacokinetic relationships should be substantially less complex when dealing with a single agent, rather than two or more.

Three are the most common strategies used for the design of MTDLs: pharmacophore linking, merging, and fusing. MTDLs can be further divided into two major categories: co-drugs and hybrids **(**Fig. 5**)**. When the two pharmacophores are joined together via a cleavable linker, the resulting MTDL can be classified as a co-drug (also named mutual prodrug), as they act as a mutual pro-moiety for each other [36]. This is due to the fact that enzymatic breakdown of the linker in the target site will produce two distinct APIs in an equimolar ratio.

The ideal criteria for a co-drug are: (a) the co-drug itself is not pharmacologically active, (b) the release of the two drugs is fast and does not produce toxic side products, (c) the linker should be biocleavable by enzymes or other cellular agents. Based on these, a co-drug is more efficient in terms of delivering higher concentrations of the two parent drugs in both plasma and brain when compared to the equimolar physical mixture of the two parent drugs [37]. It is also noteworthy that oral administration of a co-drug produces the two parent drugs in equimolar ratio, while the physical mixture may fail. This can be due to divergent pharmacokinetics of the two drugs or competition for specific uptake or efflux proteins. In addition, codrugs are generally more lipophilic than their parent compounds, increasing the likelihood of crossing cell membranes if they remain intact prior to cellular entry [38]. This strategy is typically employed to improve the pharmacokinetic properties of the parent compounds, as well as increase their safety and selectivity by rendering them inactive until metabolic cleavage.

Conversely, hybrid molecules resulting from the merging, fusing, or linking of two pharmacophores via non-cleavable linkers constitute a single chemical entity that is capable of acting on multiple targets without undergoing further biochemical modifications **(**Fig. 5**)** [36]. The ideal MTDL, apart from the essential physicochemical and drug-like properties, should also possess a balanced activity profile against its targets, while being devoid of interactions with off-targets [27]. In other words, it should be "selectively non-selective.".

Polypharmacology is not a novel concept, and its suitability in tackling complex disorders and enhancing the clinical efficacy is well

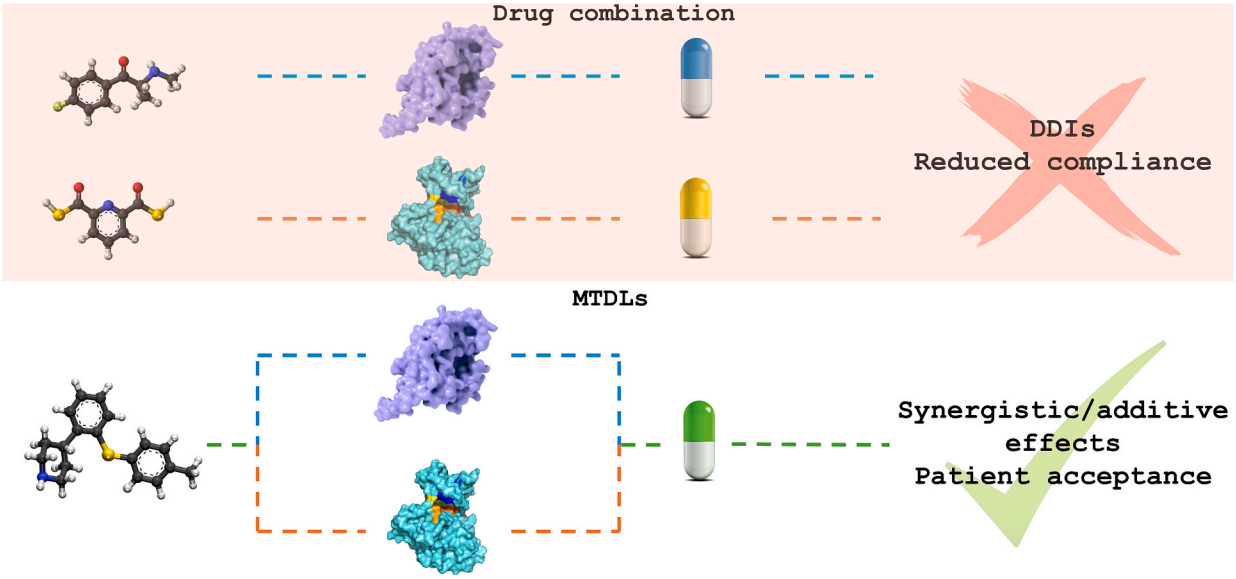

Fig. 4. Benefits of MTDLs in comparison to drug combinations.

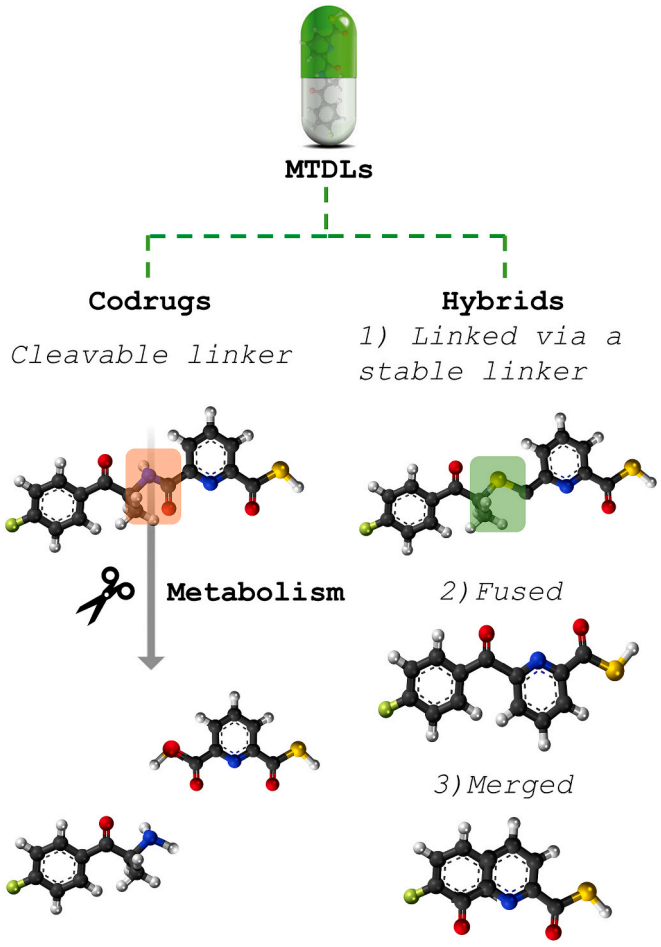

Fig. 5. The two categories of MTDLs: co-drugs and hybrids.

documented [8,39]. MTDLs have been approved for neurodegenerative diseases. Notably, rasagiline and safinamide act on multiple pathways by offering greater benefits to PD patients [40]. By contrast, the therapeutic relevance of polypharmacology for targeting CC is only partially reflected, with a few purposefully designed MTDLs entering clinical trials, and some even marketed. Examples include the dual orexin receptor antagonists (DORA) for the treatment of insomnia, as more effective for sleep promotion than antagonism of either receptor alone [4]. Advancements in our understanding of CC networks in different diseases, as well as in new techniques such as artificial intelligence and machine learning [41], have the potential to expedite the design and development of novel, safer, and more efficacious polypharmacology tools.

Before discussing how combination therapies can be employed to enhance the pharmacokinetic profile, improve selectivity, and guide drug delivery of CNS-directed therapeutic agents to the brain (**Section 4.3**), a brief overview of the BBB physiology and transport mechanisms is provided.

4. The Blood-Brain Barrier: structure, function, and transport mechanisms

4.1. Overview of the BBB

The BBB constitutes a complex and dynamic interface between peripheral tissues and the brain, tightly regulating the exchange of endogenous molecules, as well as therapeutics. Historically, the first known observations of a restriction in the exchange of molecules between the CNS and the periphery were made by Ridley in 1695 [42]. In 1885, Paul Ehrlich was the first to demonstrate this phenomenon experimentally by injecting rabbits with an acidic dye and observing the specific exclusion of the dye from the CNS. However, the notion of a physical barrier in the cerebral vasculature was proposed fifteen years later by Lewandowsky, while robust evidence for the existence of the BBB was provided by Goldman in 1913 [42].

Anatomically, the most elemental components of the BBB are brain endothelial cells (BECs), characterized by the presence of tight and adherence junctions that seal the paracellular gaps, severely limiting the number of substances that reach the brain. The formation and function of the BBB are supported by pericytes and astrocytes, which are in constant communication with extracellular matrix components, glial cells, and neurons. Collectively, this dynamic system is referred to as the neurovascular unit **(**Fig. 6**A)** [10].

4.1.1. Endogenous transport mechanisms

Although heavily restricted, the brain is not an isolated organ. The bidirectional flux of molecules occurs perpetually, through gradient-driven passive mechanisms or active, energy-consuming means. Paracellular transport, defined as the passage of molecules through the gaps between BECs, is relevant only to small, water-soluble compounds. However, it is severely hampered by the existence of tight junctions (TJs) and efflux pumps. Conversely, the majority of CNS drugs rely on the transcellular diffusion pathway, given their physicochemical properties. Generally, CNS-directed agents are lipophilic compounds with a molecular weight (MW) < 450 Da, tPSA (total polar surface area) < 90 $Å^2$ and a maximum of three hydrogen bond donors. These properties increase the likelihood of accumulating in the brain, unless they are substrates for efflux pumps [43].

Essential proteins and other macromolecules can reach the brain parenchyma by receptor-mediated transcytosis (RMT), which involves the binding of the molecule to a specific receptor expressed in the BBB luminal side, followed by invagination of the membrane and encapsulation of the receptor-ligand complex into an intracellular vesicle. These vesicles are driven towards the basolateral membrane of the cells and can fuse with endosomes, leading to the exocytosis of the ligand and recycling of the receptor. Through a similar mechanism, initiated by electrostatic interactions, cationic molecules, such as albumin, can gain access to the brain via absorptive-mediated transcytosis. Finally, hydrophilic nutrients, such as glucose or specific amino acids, cross the BBB by carrier-mediated transport (CMT), mainly utilizing solute-like carriers (SLCs) [44] **(**Fig. 6**B)**.

4.2. BBB alterations in brain disorders

Compelling evidence indicates BBB breakdown as the instigating factor or an early biomarker for CNS diseases. Degeneration of BECs and pericytes results in the loss of BBB integrity, disruption of TJ function, and increased paracellular and/or transcellular flow of solutes. In parallel, dysregulation of efflux transporters aids in the accumulation of neurotoxic agents. For example, in AD, high concentrations of Aβ can be in part attributed to the decreased clearance, stemming from P-glycoprotein (P-gp) downregulation in the diseased BBB [45]. Moreover, the increase in BBB permeability can lead to higher influx of pro-inflammatory mediators and ROS production, resulting in neuronal damage. BBB disruption (BBBD) has been observed in depressed patients, with stress and inflammation playing a critical role in the loss of BBB integrity [46]. Moreover, alterations of TJ have also been detected in glioblastoma, resulting in extensive edema [47]. The distinct characteristics of the diseased BBB are crucial and should be considered, since they can alter the pharmacokinetic profile of a drug and thus the treatment outcome. Additionally, since its dysfunction can be reduced or reversed, the BBB has been proposed as a therapeutic target for brain conditions. Preserving or restoring its barrier-like properties and minimizing leakage may represent valuable strategies in the fight against neurodegeneration [48].

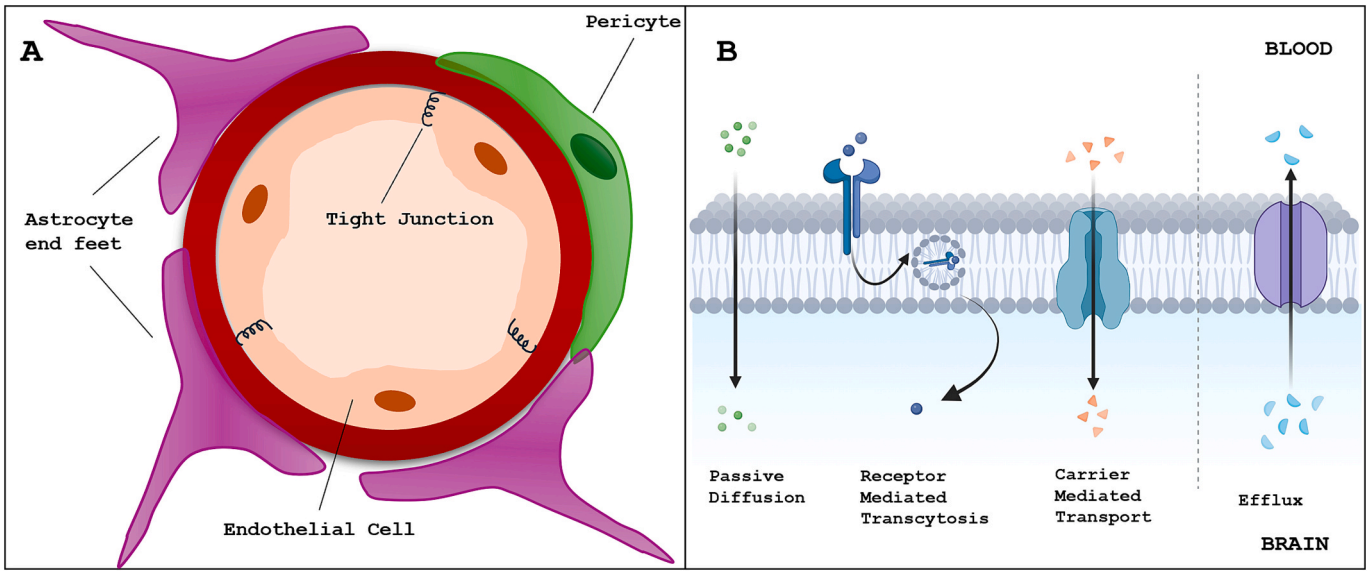

Fig. 6. (A) Cross-section of the neurovascular unit with its basic anatomical features. Brain endothelial cells, characterized by the presence of TJs, line the inside capillary walls, surrounded by pericytes and the end feet of astrocytes. (B) Mechanisms of transcellular transport of molecules across the BBB.

4.3. BBB: A barrier or a carrier for CNS drug delivery?

The exchange of molecules between the brain and the systemic circulation is tightly regulated by the BBB. This results in the inability of over 98% of small molecule drugs to reach significant brain concentrations, while none of the biological agents (antibodies, recombinant proteins, etc.) can enter the brain without an appropriate delivery system, such as liposomes or nanoparticles [42]. This phenomenon represents the biggest hurdle in drug development against brain pathologies. However, the BBB, apart from being a *barrier,* can also act as a *carrier*, due to its ability to transport the necessary nutritional molecules, like amino acids and glucose, from the blood to the brain tissue. Thus, a plethora of strategies have been implemented to overcome its barrier-like features, while simultaneously exploiting its carrier-like functions. Even though there is a wide variety of approaches, such as simple chemical modifications to the API [49], physical BBBD using focused ultrasound and microbubbles [50], and advanced drug delivery systems [51], polypharmacology may play a crucial role in enhancing brain uptake of CNS-directed drugs. As depicted in Fig. 7, polypharmacology may be exploited in three different ways:

1) By manipulating BBB integrity (i.e., by opening BBB junctions) **(**Fig. 7**A);**
2) By blocking BBB efflux systems **(**Fig. 7**B);**
3) By harnessing BBB-specific transport systems **(**Fig. 7**C-D).**

Although no specific examples directed to CC have been reported, the development of co-drugs and targeted prodrugs may be a valuable strategy for enhancing brain delivery of drugs targeting CC for the treatment of CNS disorders. In the following, Table 1 summarizes the different combination strategies for the manipulation of BBB integrity, which are individually described in **Section 4.3.1**. Similarly, BBB efflux inhibition and BBB-specific transport systems harnessed via polypharmacology are outlined in Table 2 and are presented in a more detailed fashion in **Sections 4.3.2 and 4.3.3**.

4.3.1. Polypharmacology to enhance brain uptake of CNS-directed drugs by manipulating BBB integrity

Co-administration of hyperosmolar agents

Direct and transient loss of BBB integrity and thus an increase in the rate of paracellular transport to the brain can be achieved by the concurrent administration of a hyperosmolar agent, such as mannitol **(**Fig. 8**)**, along with the API. Proposed over 50 years ago by Rapoport *et. al.* [52], the intra-arterial (IA) infusion of hyperosmotic mannitol solutions has been a well-established and clinically relevant method of increasing the influx of CNS therapeutic agents to the brain. By inducing vasodilation along with osmotic dehydration and subsequent shrinkage of cerebrovascular endothelial cells, mannitol administration results in the disruption of TJs, leading to the widespread and non-specific formation of paracellular gaps [53]. Researchers have successfully utilized this strategy to enhance the brain uptake of drugs in animal models such as mice [54], rats [55], and dogs [56], as well as human subjects in clinical trials. In a Phase I trial, 13 patients with temozolomide-refractory oligodendroglioma or oligoastrocytoma received a therapeutic regimen of IA carboplatin and melphalan, intravenous etoposide phosphate, after IA administration of 25 % mannitol [57]. Overall, in the case of malignant brain tumors, several Phase I and II studies have been conducted [58–61], indicating mannitol as a promising addition to chemotherapeutic regimens, with a favorable risk-to-benefit ratio **(**Table 1**)** [62].

However, the unselective BBBD can potentially lead to detrimental side effects such as edema, aphasia, hemiparesis, and seizures [63,64], due to the uncontrolled influx of circulating compounds, as well as excessive amounts of fluids into the brain. This fact, together with the invasive nature of the method and the inconsistencies reported in the duration and degree of BBBD [65], has limited its application and clinical relevance only in the case of aggressive brain tumors.

Co-administration of bradykinin analogues

Bradykinin is a nonapeptidic hormone, member of the kallikrein-kinin system, with a well-established role in inflammation and vasodilation [66]. It exhibits its effects upon activation of two G-protein coupled receptors (GPCR), B1R and B2R. The former is absent in normal tissues but rapidly upregulated during inflammatory conditions, exerting a crucial role in inflammation and nociception. The latter is constitutively expressed and is involved in the dilation of blood vessels and tissue permeability [66]. B2R is also expressed in the BECs and, upon activation, induces a decrease in cAMP production, leading to the transient opening of TJs [67]. Thus, bradykinin and its analogues targeting B2R could enhance the brain bioavailability and efficacy of CNS diagnostics and therapeutics.

Bradykinin's short half-life and the absence of selectivity towards B2R instigated the development of analogues [68]. Cereport (also

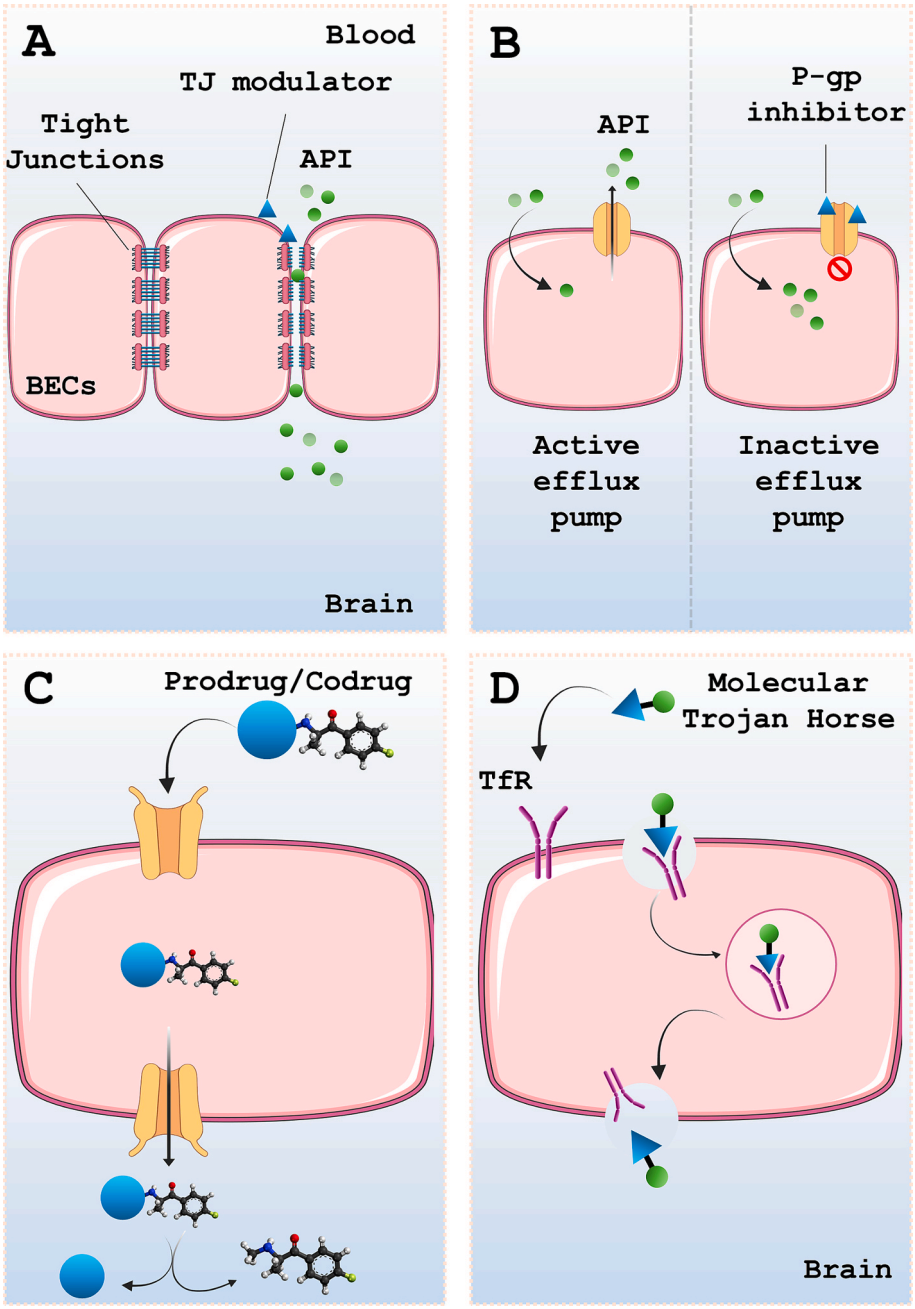

Fig. 7. Passive and active transport mechanisms in the CNS and strategies to enhance brain permeability.

known as RMP-7 or labradimil) is a nonapeptide bradykinin analogue, specifically designed to address these issues by having unnatural amino acids in susceptible positions to degradation, as well as a reduced peptide bond between amino acids 8 and 9 [68,69]. Studies in animal models have confirmed the ability of cereport to enhance the accumulation of drugs into the brain [70], particularly in the case of brain tumors [71]. In addition, no adverse effects were reported, except for a decrease in blood pressure [72].

Results from a Phase I clinical trial confirmed that a carboplatin and cereport regimen was well-tolerated with only minimal side effects (Table 1) [73,74]. Unfortunately, subsequent Phase II studies failed to demonstrate the efficacy of cereport in combination with carboplatin in patients with malignant glioma [75].

Co-administration of alkylglycerols

Alkylglycerols (AKGs) are glycerol ether lipids, abundantly found in shark liver oil, which has traditionally been used in Scandinavian folk medicine for the treatment of cancer and various other CNS diseases [76]. To date, AKGs can be found commercially in food supplements, while there are indications that they possess antiproliferative and cytoprotective properties [76,77]. In the case of BBBD, seminal work has been done by Erdlenbruch and co-workers [78–81], who demonstrated that AKGs can reversibly disrupt the integrity of the BBB (Table 1). The authors demonstrated that intracarotid (IC) administration of 1-O-pentylglycerol (Fig. 8) greatly enhanced the brain uptake of anti-tumor agents such as methotrexate (230-fold) and *cis*-platin (125-fold). On the contrary, for larger molecules (vancomycin and gentamicin), brain accumulation was increased to a lesser extent (15- and 12-fold respectively), indicating a certain degree of size bias [78]. Subsequent studies revealed several advantages, such as the considerably shorter period of BBBD compared to that of mannitol, the increased effectiveness in comparison to bradykinin analogues, and the low systemic toxicity [79–81]. Moreover, AKGs exhibit a favorable pharmacokinetic profile in

Table 1
Comparison of combination strategies to manipulate BBB integrity.

Strategy	BBBD Mechanisms	Advantages	Drawbacks	Ref.
Hyperosmotic agents	Osmotic shrinkage of BECs, vasodilation	Clinically approved, Short duration of action	IA administration, No size-dependence, Side effects	[50–63]
Bradykinin Analogues	Transient opening of TJs	Low systemic toxicity	No efficacy in clinical trials, IC administration	[64–73]
Alkylglycerols	Internalization of TJ proteins	Low systemic toxicity, Size-dependence, Short duration of action	IC administration, Preclinical evidence	[74–83]
Borneol	Downregulation of TJs and efflux pumps	Oral administration, GRAS status, BBB protective effects	Preclinical evidence	[84–91]
Regadenoson	Downregulation of TJs and efflux pumps through $A_{2A}R$ agonism	Oral administration, Clinically approved for other indications	Preclinical evidence	[92–96]
Sodium Caprate	Disruption of TJ formation and function	GRAS status	IC administration, No size-dependence, Preclinical evidence	[97–100]
Fingolimod	Downregulation of S1PRs	Oral administration, Size-dependence, Clinically approved for other indications	Preclinical evidence	[101–104]
OKN-007	Unknown	Oral administration	Preclinical evidence	[105–107]

Abbreviations.
IA, intraarterial; IC, intracarotid; BBBD, Blood-Brain Barrier Disruption; GRAS, Generally Regarded as Safe; TJ, Tight Junction.

Table 2
Polypharmacological strategies to enhance the brain uptake of CNS therapeutics.

Strategy	Mechanism	Limitations	Ref.
Inhibition of efflux	Inhibiting efflux pumps allows the accumulation of pharmaceuticals in the brain	Side effects, lack of efficacy in clinical trials	[108–115]
CMT	Designing prodrugs with structural similarity to SLC substrates	Preclinical evidence	[116–128]
RMT	Transcytosis by hijacking endogenous receptors	Not approved yet	[129,130]

Abbreviations.
BBBD, Blood-Brain Barrier Disruption; CMT, Carrier-Mediated Transport; SLC, Solute Carrier; RMT, Receptor-Mediated Transcytosis.

vivo, since the majority of 1-O-pentylglycerol was found in urine after 4.5 h, with no evidence of tissue accumulation [82]. The promising preclinical results are overshadowed by the invasive nature of the procedure, prompting the exploration of alternative ways to utilize AKGs, such as their incorporation into the surface of nanoparticles [83,84].

Although the exact mechanism remains unclear, *in vitro* studies on BEC cultures indicate that BBBD is caused by redistribution and internalization of TJ proteins like claudin-5 and alterations in cell shape, thus enhancing paracellular transport [85].

Co-administration of borneol

Borneol (Fig. 8) is a lipid-soluble bicyclic monoterpene, naturally occurring in plants such as *Dryobalanops aromatica* and *Cinnamonum camphora* [86]. It has been widely used in traditional Chinese medicine for more than a thousand years as a remedy for a plethora of diseases, as well as a "carrier" that delivers other APIs to the brain [86]. In Western medicine, it is considered a GRAS (Generally Regarded As Safe) compound by the FDA and is used as a fragrance in cosmetics and a food additive, while its neuroprotective, vasodilating, anti-nociceptive, and anti-inflammatory properties are still being investigated [87].

It has been reported that borneol can improve the brain uptake of various therapeutics such as cisplatin [88] and kaempferol [89], while also promoting the brain accumulation of nanoparticles [90]. The observed increase in BBB permeability is achieved by two main mechanisms. Firstly, as in the case of alkylglycerols, borneol leads to the internalization of TJ proteins (claudins and occludins) within 30 min of administration, lasting for up to 8 h (Table 1) [91]. Secondly, it downregulates the expression of efflux pumps such as P-gp and other ATP-binding cassette (ABC) transporter proteins in rats [92]. Notably, borneol's effect appears to be regiospecific, enhancing the uptake of small molecules mostly into the hippocampus and hypothalamus [92].

Apart from the opportunity to deliver therapeutic interventions to specific brain regions, the co-administration of borneol possesses significant advantages in comparison with other strategies. Firstly, it is administered orally, making it a non-invasive method, which can

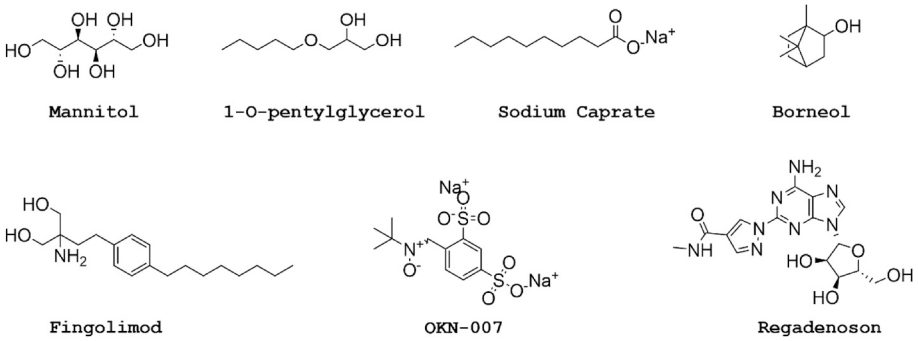

Fig. 8. Chemical structures of small molecules capable of manipulating BBB integrity.

significantly improve patient compliance, particularly in chronic conditions. Moreover, borneol exerts a protective effect on the BBB integrity in pathological conditions such as ischemic stroke, reducing its permeability and preserving its barrier properties [93]. Despite these promising data, further research is needed to validate the safety and efficacy of borneol in humans in a randomized and controlled setting.

Co-administration of regadenoson

Regadenoson **(**Fig. 8**)** is an FDA-approved adenosine receptor 2A ($A_{2A}R$) agonist, commonly used as a vasodilator in cardiac stress tests. It is able to transiently cause BBBD in rats [94] and *in vitro* human BBB models [95]. The associated mechanism of action is similar to that of borneol, as $A_{2A}R$ activation leads to cytoskeletal reorganization and downregulation of TJs and efflux pumps **(**Table 1**)** [95,96]. Unfortunately, human studies failed to replicate these encouraging results, halting its further development [97]. However, a promising alternative could be the incorporation of regadenoson into nanoparticles or other drug delivery systems, as in the case of AKGs [98].

Co-administration of sodium caprate

Sodium caprate **(**C10, Fig. 8**)** is a medium-chain fatty acid commonly used as a food additive with a GRAS FDA status, as well as in pharmaceutical formulations in Sweden and Japan [99]. It has been systematically studied in clinical trials as an intestinal permeation enhancer for increasing the oral bioavailability of macromolecules [99]. IC administration of C10 has been indicated to exert a reversible BBBD in rats [100] and sheep [101], induced by directly interacting with claudins and thus disrupting TJ formation **(**Table 1**)** [102]. However, the high invasiveness of this approach has limited further studies.

Co-administration of fingolimod

Sphingosine 1-phosphate (S1P) is an endogenous signaling molecule involved in anti-apoptotic, inflammatory, and cell replication pathways by activating a group of five GPCRs called S1PR1–5 [103]. Fingolimod **(**FTY720, Fig. 8**)** is an approved, orally administered immunomodulatory agent prescribed for the treatment of relapsing-remitting multiple sclerosis (MS). It exerts its effects through the non-selective agonism of S1PRs, which eventually leads to their internalization and downregulation [104]. Since S1PR1 regulates the localization of TJs, fingolimod can disrupt BBB integrity and allow the paracellular transport of molecules in a size-dependent manner **(**Table 1**)** [105]. These findings are also corroborated by a recent clinical trial, examining the effects of concurrent administration of fingolimod and the thrombolytic agent alteplase in acute ischemic stroke patients [106]. The 66 participants were split equally into two groups, one receiving alteplase alone and the other a combination of fingolimod with alteplase. The second group showed a statistically significant improvement in the efficacy of alteplase treatment after 24-h, as well as superior functional recovery after 90 days. Although this study has several limitations, i.e., the small sample size and the open-label protocol, further investigations are warranted [106].

Co-administration of OKN-007

OKN-007 **(**Fig. 8**)** is a low-MW nitrone compound currently being investigated in clinical trials for the treatment of high-grade glioma and glioblastoma [107]. It was recently reported that following its administration in mice, there is a transient BBB opening, allowing the influx of compounds ranging from 550 Da to 450 kDa in the brain **(**Table 1**)** [107]. In addition, its synergistic effects with temozolomide (TMZ) in reducing tumor growth were elucidated using human G55 xenograft models [131]. Currently, the efficacy and safety of a combination of OKN-007 and TMZ against recurrent malignant glioma are being evaluated in a multicenter Phase II clinical trial [132].

4.3.2. Drug combinations to inhibit the efflux transporters of CNS-directed drugs

The ability of drugs to translocate into the brain is severely limited by the presence of efflux transporters. In mammals, most efflux transporters belong to the ATP-binding cassette (ABC) transporter family **(**Fig. 7**B)** and include P-gp and other multidrug-resistance proteins [108,109]. Abundantly expressed in the BBB, P-gp is the most widely studied and clinically relevant efflux transporter and constitutes a major hurdle in the successful delivery of CNS-directed therapeutics into the brain. It is a membrane-bound molecular pump that actively exports drugs and various endogenous or exogenous substances out of the brain by consuming ATP. Hence, co-administration of a P-gp inhibitor has been proposed as a potential strategy to increase the brain accumulation of CNS therapeutic agents **(**Table 2**)** [108,109]. For example, the antidepressant effect of escitalopram is severely limited by its extrusion from the brain due to its affinity for P-gp. By co-administering the drug with a P-gp inhibitor in rodents, O'Brien et. al. [110] noted an increase in escitalopram's brain levels that was translated to an increased pharmacological effect. The authors suggest the co-administration of a P-gp inhibitor as a viable option to enhance the therapeutic efficacy of escitalopram [110]. Since numerous drugs or drug candidates are P-gp substrates, significant research efforts have been directed towards its inhibition. To this end, four generations of P-gp inhibitors have been developed. The first comprises already approved drugs with a distinct mechanism of action that were also found to modulate P-gp activity, such as verapamil and tamoxifen. Owing to their low affinity for P-gp, high doses were required for its modulation, leading to serious side effects that prohibited their clinical use for this purpose [111]. The second generation was developed by introducing chemical modifications to the first generation, with the objective of retaining the P-gp inhibitory effect, while diminishing the pharmacological activity of the parent compounds. Similarly, adverse effects stemming mostly from interactions with CYP450 halted their clinical utilization [112]. The *de novo* development of compounds specifically designed to modulate the activity of P-gp while minimizing off-target effects gave rise to the third generation of inhibitors, including laniquidar, tariquidar, and elacridar. These molecules have been demonstrated to effectively enhance drug uptake into the brains of rats [113], mice [114], and non-human primates [115]. P-gp inhibition by tariquidar has been observed in human studies through the use of positron emission tomography scans in healthy volunteers [116,117]. Notwithstanding these findings, these compounds have failed in clinical trials, although further investigation is still ongoing.

4.3.3. Co-drugs and targeted prodrugs: Exploitation of passive and active transport mechanisms for CNS-directed drugs

While small lipophilic agents may traverse the BBB passively via diffusion, this is not the case for hydrophilic molecules or macromolecules. Nevertheless, nutrients essential for brain function, including glucose, amino acids, nucleotides, and specific hormones, must cross the BBB. They do so by employing active transport **(**Fig. 6**B)**. From chemical modifications and drug conjugation to the incorporation in advanced delivery systems, polypharmacology can leverage active transport mechanisms. In the following, we describe the development of polypharmacological tools, including co-drugs and targeted prodrugs with increased brain accumulation.

Co-drugs: Making passive diffusion possible

A "co-drug" or "mutual prodrug" is a single molecule obtained by the conjugation of two therapeutic compounds with synergistic activity, via a cleavable linker [37]. Upon metabolic transformation, the two starting compounds of a co-drug have the potential to be released in the same target cells and at the same time. This contrasts with drug combinations, which are comprised of two single molecules, each with an individual pharmacokinetic profile. It should be noted, however, that the co-drug approach is only applicable to starting compounds that possess functional groups suitable for conjugation [118]. Co-drugs also differ from prodrugs since they avoid the use of an unwanted "pro-moiety" that could cause adverse effects after its release. Instead, the rational design of co-drugs is based on the synergistic effects of the two released APIs [37]. They are, essentially, masked MTDLs. This strategy has the potential to be particularly advantageous for CNS drug delivery since it can potentially address the complexity of CNS disorders while

simultaneously providing an efficient delivery system for MTDLs. For example, PD is characterized by elevated ROS levels, besides the impaired dopaminergic signaling, that severely contributes to PD progression. Pinnen et. al. developed co-drugs of L-dopa with sulfur-containing antioxidant moieties such as cysteine, methionine and bucillamine that could be beneficial against PD **(**Fig. 9**)** [119]. Co-drug **1 (**Fig. 9**)**, stemming from the conjugation of methionine and L-dopa through an amide bond, afforded higher concentrations of dopamine in the striatum than L-dopa alone. Moreover, based on data identifying α-linolenic acid (ALA) and valproic acid (VPA) as positive modulators of MS, our group recently developed ALA-VPA co-drugs to counteract the MS progression [118]. ALA and VPA were conjugated via three different linkers (ethylene glycol, ethanolamine, ethylenediamine), resulting in four co-drugs. Co-drug **2**, featuring a diamide linker **(**Fig. 9**)**, undergoes fatty acid amide hydrolase-dependent metabolism with a half-life of about 23 min in rat brain homogenates, while exhibiting high plasma stability, suggesting a brain-specific release of the parent APIs. Co-drug **2** showed better neuroprotective, remyelinating, and immunomodulatory response than each of the parent drugs or their combination, serving as a preliminary proof-of-concept for the application of this strategy in MS and possibly other neurodegenerative disorders [118].

Targeted Prodrugs: Exploiting Carrier-Mediated Transport

Classical prodrug design frequently entails the use of nonspecific chemical approaches to mask undesirable drug properties, including limited bioavailability, lack of site specificity, and chemical instability [120]. Thus, prodrugs are pharmacologically inert derivatives of an API, specifically tailored to improve the ADME properties of the parent compound or deliver it to the desired tissue. Upon doing so, the prodrug is biologically converted back to the original active moiety, which proceeds to exert its pharmacological effect. Targeted prodrug design represents a novel strategy for directed and efficient drug delivery. Prodrugs targeting a specific enzyme or a specific membrane transporter have the potential to serve as selective drug delivery systems [120]. The prodrug approach, in its simplest form, has been extensively utilized in medicinal chemistry to enhance brain delivery of compounds by masking, for example, labile –COOH groups as esters and thus increasing lipophilicity [121]. For example, L-dopa prodrugs may be employed for the treatment of PD, potentially facilitating enhanced drug influx into the brain by circumventing peripheral metabolism. This provides an alternative strategy to the concomitant administration of MAO inhibitors with fewer side effects [122].

Solute carriers (SLCs) facilitate the influx of nutrients into the brain by carrier-mediated transport (CMT). To date, only a handful of drugs, including L-dopa, melphalan, baclofen, gabapentin, and valproic acid, have been shown to gain access to the brain by mimicking the natural substrates of specific SLCs. A promising strategy is the design of targeted prodrugs, i.e., cleavable molecules consisting of a drug linked to a carrier substrate, where one part of the molecule will bind to the target of interest and the other part to the specific SLC. SLCs, such as GLUT-1 and LAT-1, are abundantly expressed in the BBB and allow the uptake of essential nutrients, e.g., glucose and certain amino acids. Thus, a potential drug candidate can be glycosylated or conjugated to an amino acid via a cleavable linker to hijack GLUT-1 or LAT-1, respectively. This approach could improve not only the brain uptake of the parent compound but also its pharmacokinetic profile [123]. Ideally, such targeted prodrugs should possess high affinity for the targeted carrier, be stable in the peripheral circulation and plasma, and should be readily hydrolyzed once in the brain to release the drug.

GLUT-1

GLUT-1 constitutes the most abundant glucose transporter in the brain, found in the luminal and abluminal membrane of BEC [124], therefore emerging as a target of interest for the design of targeted prodrugs. Bonina *et. al.* demonstrated increased brain uptake and efficacy of glycosylated, 7-chlorokynurenic acid and dopamine/L-dopa in animal models [125,126]. The glycosylated 7-chlorokynurenic acid targeted prodrug **3** **(**Fig. 10**)** exhibited high localization into the brain tissue and an increased anti-seizure effect, compared to free 7-chlorokynurenic acid [125]. Similarly, targeted prodrug **4 (**Fig. 10**)** showed good plasma stability, increased brain concentration, and activity compared to free L-dopa [126]. In recent years, this approach has been exploited in more advanced delivery systems. Notably, by incorporating mannose and cell-penetrating peptides in the surface of liposomes carrying the

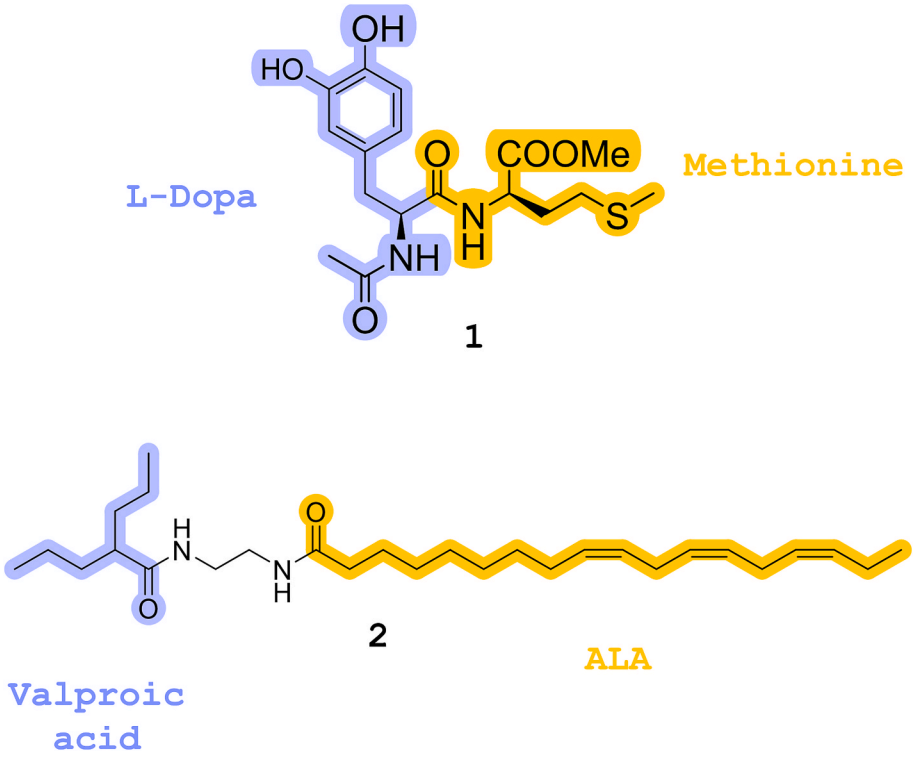

Fig. 9. Examples of the co-drug strategy.

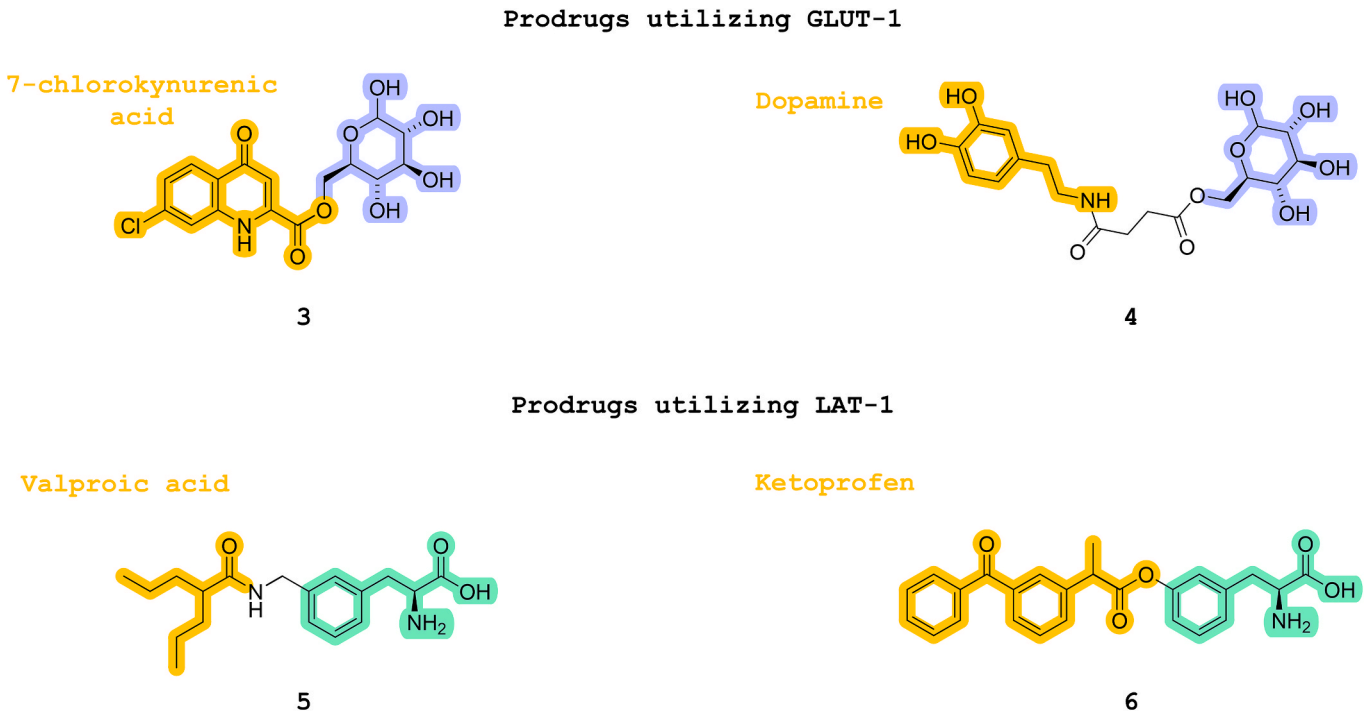

Fig. 10. Examples of prodrugs utilizing CMT to enter the brain.

BDNF (brain-derived neurotrophic factor) gene, a significant increase in the uptake of the conjugated liposomes has been demonstrated compared to unconjugated ones in in vitro and in vivo models [127]. The objective was to ascertain whether the negligible expression of GLUT-1 in tissues other than the BBB could be exploited for targeted delivery. To this end, mannose was employed as a targeting moiety rather than a permeation enhancer to improve brain delivery [127].

LAT-1

L-type amino acid transporter 1 (LAT-1) is a heterodimeric SLC located in the membrane of BECs that efficiently transports L-amino acids (e.g., phenylalanine) into the brain. As previously mentioned, approved CNS-directed drugs (e.g., melphalan, gabapentin, L-dopa, etc.) traverse the BBB via this carrier, owing to their close structural resemblance to its natural substrates.

Seminal work in LAT-1 targeted prodrugs has been performed by Huttunen *et. al.*, who have successfully demonstrated the versatility and significance of this approach [128]. By conjugating valproic acid [128] or ketoprofen [133] to an aromatic amino acid (prodrugs **5** and **6** respectively, Fig. 10), the authors developed LAT-1-targeted prodrugs exhibiting improved pharmacokinetic properties and increased brain permeability. Utilizing such prodrugs might prove beneficial in CNS disorders since the expression of LAT-1 in glial cells, neurons, and astrocytes allows drugs to not only surpass the BBB and access the cerebral circulation, but also to accumulate inside brain cells. Moreover, contrary to other transporters such as GLUT-1, the expression of LAT-1 remains stable even during neurodegenerative conditions, e.g., AD [134].

Targeted Prodrugs: Hijacking Receptor-Mediated Transcytosis

Nutrients such as leptin, insulin, and iron can enter the brain by binding to specific receptors expressed in the luminal and/or the abluminal membrane of the BBB through receptor-mediated transcytosis (RMT, Fig. 7**D**). This transport mechanism can be exploited in the development of novel BBB-permeant therapeutics by attaching a pharmacophore onto an RMT delivery vector, i.e., a natural or synthetic moiety capable of triggering the RMT process. These vectors, also termed "molecular trojan horses" can effectively transport drugs, including small molecules and biologics, into the brain parenchyma [135].

Transferrin Receptor (TfR)

TfR is the most established receptor involved in RMT. Iron is typically bound to a glycoprotein called transferrin while in circulation. Its accumulation into the brain is mediated and tightly regulated by the TfR. The choice of the TfR ligand in TfR-targeted prodrugs encompasses both the endogenous ligand together with different antibodies and peptides that bind to different epitopes of the receptor molecule. Although conjugation to transferrin is not an ideal strategy for CNS drug delivery due to the competition with the natural substrate, monoclonal antibodies, such as OX26, that bind to distinct epitopes have exhibited promise in preclinical settings [136]. In addition to endogenous transferrin or antibodies, small peptides that bind to the TfR have been developed [129]. The advantages of such small peptides are that the resulting prodrug does not compete with the endogenous transferrin for binding the receptor, and that potential side effects of the antibody are avoided. However, peptides are known to be very unstable in plasma, which may make them less relevant compared to antibodies. In addition to the TfR-targeting molecule, the choice of the CNS-directed agent and the attachment points where the cleavable (e.g., redox/acid −sensitive linkers) or stable linkers are incorporated to enable the release of the therapeutic agent from the targeting ligand or BBB transcytosis, are crucial aspects in the design of TfR-targeted drugs. Noteworthily, the TfR-targeting ligand needs to dissociate from the receptor during endosomal sorting process, because a slow TfR dissociation rate and a too high affinity trigger TfR lysosomal degradation. Several such TfR-targeted drugs have been tested in clinical trials or are currently under clinical evaluation [130].

The large polypeptide nerve growth factor (NGF) conjugated to an anti-TfR antibody for the treatment of AD has proven the possibility of transporting larger molecules like proteins across the BBB by exploiting TfR-mediated transcytosis. NGF is not able to penetrate the BBB, which makes its clinical utility dependent on invasive neurosurgical procedures. When conjugated to the TfR-directed OX26 antibody via a cleavable disulfide linker, TfR-mediated transcytosis enables NGF to cross the BBB. This TfR-targeted NGF increased the survival of both cholinergic and noncholinergic neurons [137]. Additionally, this approach may apply to the treatment of other brain disorders with other BBB-

impermeable therapeutic agents.

Trontinemab, developed by Roche, is the first TfR1-directed Brainshuttle™ coupled to an Aβ antibody (gantenerumab) [138] for the treatment of AD to enter clinical trials (NCT04023994 and NCT04639050). Results from a Phase III trial of gantenerumab and other amyloid-directed antibodies suggested exposure-dependent issues on amyloid and cognitive decline. Trontinemab has been designed as a 2 + 1 bispecific monoclonal antibody, consisting of a bivalent anti-amyloid antibody (gantenerumab), conjugated to a monovalent fragment that binds to the TfR via a stable linker. Trontinemab's 2 + 1 format allowed a monovalent binding to human TfR1 in systemic circulation, whilst preserving the bivalent binding of Aβ plaques in the brain. In addition, the 2 + 1 format is associated with lower systemic adverse effects as reported for other TfR1-targeted drugs. Remarkably, trontinemab presents a 50-fold increase in brain uptake compared with gantenerumab [138]. The modular nature of the 2 + 1 Brainshuttle™ format of trontinemab holds potential for the application of the TfR1 shuttling technology to other CNS-directed antibodies.

5. Polypharmacology to target the CC

5.1. The circadian clock and the BBB

As discussed previously, the CC regulates a plethora of internal processes in a periodic manner as a response to light/dark stimuli. Interestingly, BBB function and permeability also exhibit diurnal oscillation, as demonstrated by the time-dependent fluctuation in the brain levels of tumor necrosis factor alpha, norepinephrine, leptin, and delta sleep-inducing peptide [139]. Moreover, P-gp activity is decreased during the resting phase, resulting in an increased accumulation of P-gp substrate drugs in animal models. In *period* null (*per*^{01}) flies, which lack proper CC functions, no oscillation was observed, providing further evidence for the circadian control of BBB permeability [139]. The proposed mechanism for this phenomenon does not involve the direct regulation of P-gp expression by CC components but is rather driven by alterations in the levels of free intracellular Mg^{2+}. During the active phase, higher concentrations of Mg^{2+} induce the activity of efflux transporters, while in resting periods, lower levels of Mg^{2+} result in the subsequent decrease of BMAL1 and P-gp activity, thus leading to the accumulation of xenobiotics in the brain [140]. Furthermore, TJ formation and function are subject to CC regulation, as demonstrated by the time-dependent variation in the expression of key proteins such as occludin and claudin-5 [141].

The diurnal alterations in BBB permeability can have a profound effect on the management of brain disorders. For example, nocturnal administration of the antiepileptic agent phenytoin was shown to be more effective and less toxic when compared to daytime intake [142]. The concept of aligning the administration of CNS-directed therapeutics with the CC of the patient is called chronotherapy.

5.2. Chronotherapy

In recent years, there has been growing evidence that the concept of *chronos* (time) has a significant impact on the efficacy of a therapeutic intervention. This has led to the emergence of disciplines such as *chronobiology*, which studies biological rhythms and oscillating events in living organisms [143]. Chronotherapy involves aligning the intake of medication to the appropriate circadian time. Although it is one of the "five rights" of drug administration (the right patient, the right drug, *the right time*, the right dose, and the right route) [144], its importance has been severely underestimated. Unlike light therapy or CC modulators that aim to directly modulate the circadian system, chronotherapy does not involve a direct action but rather exploits the timing of the intervention to produce the desired therapeutic outcome or to minimize the side effects [145]. Over 80 % of genes coding for proteins characterized as "druggable" by the FDA display diurnal oscillations [146]. Indeed, the majority of drug target proteins are encoded by rhythmically expressed genes [147]. Consequently, time-dependent alterations in pharmacological effects have been demonstrated for over 300 drugs in human studies [148]. Thus, applying the principles of chronotherapy may result in an increase in therapeutic efficacy and/or the minimization of side effects. When dealing with diurnal fluctuations in drug efficacy, two critical factors must be considered: (1) circadian changes in drug absorption, distribution, metabolism, and overall bioavailability (chronopharmacokinetics); and (2) circadian changes in the expression of drug receptors or signal transduction pathways (chronopharmacodynamics). Additionally, time-dependent changes in toxicity and adverse side effects must also be considered (chronotoxicity). Together, these factors form the basis of chronopharmacology, which focuses on designing and evaluating treatments that release APIs by aligning with the body's biological needs. As a result, chronotherapy emphasizes using the patient's biological clock and the disease's progression to optimize therapeutic outcomes while minimizing unwanted side effects [149]. For example, the impact of dosing time on the pharmacological activity of various antidepressants targeting serotonergic, noradrenergic, and/or dopaminergic neurons was examined [150]. All antidepressants demonstrated a reduction in immobility of C57BL/6 mice in the tail suspension test, yet their activities exhibited variability according to the dosing time. Fluoxetine and imipramine induced relatively robust rhythms with high amplitudes, showing their maximal effects in the morning and evening, respectively. Venlafaxine and bupropion induced weak rhythms with maximal effects in the evening and dawn, respectively. These findings indicate that antidepressant efficacy is linked to circadian fluctuations and that antidepressants with different mechanisms of action exhibit distinct chronopharmacological profiles.

5.3. Targeting circadian clock dysfunctions

Opportunities for CC polypharmacology: The future?

In the last few years, there has been growing interest in circadian pharmacology, based on the increasing importance of circadian rhythmicity in pathophysiology. Deciphering the mechanisms of CC dysfunctions in diseases and discovering potential interventions to delay or halt disease progression will advance our ability to extend health span, treat CC-related pathology, and improve quality of life.

As discussed in recent reviews [151–153], research efforts have afforded a myriad of compounds targeting specifically a single node of the CRD network, with varying degrees of success. Although these examples demonstrated the suitability of single-target agents to modulate CC, it is unlikely that monotherapy will be effective against the complex CRD network. Combined interventions and MTDLs may offer additive or synergistic effects and are likely to be more effective than monotherapy. Nevertheless, the potential of polypharmacology remains untapped, although a few examples (mainly combinations) do exist in literature, as described in the following paragraphs. We advocate for the exploitation of polypharmacological tools against CRD in brain disorders based on our group's expertise in the field, as well as the complexity and intertwining pathways characterizing the clock's molecular machinery.

Combinatorial interventions in CC

Combined interventions may additively or synergistically increase their effectiveness on CRD, depending on the fact that the combined drugs act within the same pathway or modulate two targets belonging to different signaling pathways, respectively. Despite the limited data on the effect of combined interventions on CC, the concurrent use of drugs and non-pharmacological treatments has been explored against CRD in brain diseases (Table 3). Exogenous melatonin is commonly prescribed against sleep disorders, but is often limited by a lack of efficacy [154]. On the contrary, melatonin administration combined with light therapy seems to provide better outcomes than either treatment alone, although more robust data are needed [155]. Moreover, the combination of melatonin with the standard anti-AD drug memantine has been shown to be more effective than each treatment alone in animal models [156].

Table 3
Polypharmacological tools against CRD.

Combinatorial Intervention	Outcome	Ref.
Melatonin + Light therapy	Better results than each individual treatment	[155]
Melatonin + Memantine	Decrease Aβ aggregates and increase episodic memory	[156]
SR9011 + KL001	Synergistic antiproliferative effects	[157]
Agomelatine	Improve antidepressant effect through modulation of serotonin and melatonin receptors	[158]

The synergistic action of these interventions significantly decreased the number of Aβ aggregates and enhanced episodic memory to a greater extent compared to the separate administration of each drug. One of the proposed mechanisms of action of melatonin is the inhibition of glycogen synthase kinase-3β (GSK3β) and cyclin-dependent kinase 5 (CDK5), both of which are closely linked to CC dysfunctions [156]. It was recently demonstrated that in glioblastoma stem cells, which are heavily reliant on BMAL1 and CLOCK expression for tumor maintenance and progression, the concurrent administration of SR9011 (REV-ERB agonist) and KL001 (CRY agonist) induced synergistic antiproliferative effects [157].

Multi-Target-Directed Ligands

Unfortunately, no MTDLs have been deliberately developed to target the CC, although there are some examples of polypharmacological CC modulators, retrospectively discovered to act on the CC.

Agomelatine, a clinically used antidepressant, is a 5-HT2B/2C serotonin receptor antagonist, but also acts as an agonist on CC-modulating melatonin receptors. Thus, besides its antidepressant effect, it has been shown to increase non-REM sleep and improve sleep quality in depressed patients, which is strongly linked to improved antidepressant outcomes (Table 3) [158]. This dual effect can be attributed to the close structural resemblance of agomelatine with serotonin and melatonin, and the fact that both receptors belong to the GCPR superfamily. Nevertheless, it is evident that positively impacting CC function may provide greater clinical benefits in several brain disorders.

6. Non-pharmacological options against CRD-related diseases

Chemistry and biotechnology are the pillars of modern medicine, as we rely almost exclusively on pharmaceutical agents to treat and prevent diseases. New regulations are constantly being imposed to drive the development of more advanced, effective, and safe treatments. It is worth noting, however, that non-pharmacological options, such as cognitive behavioral therapy (CBT), show great promise for conditions such as insomnia and sleep disorders [159]. As already mentioned, disturbances in the sleep-wake cycle are involved in neurodegeneration, which supports CC as a promising point of intervention to combat neurodegenerative disorders such as AD or PD. Non-pharmacological options such as sleep hygiene, CBT, bright light therapy, and continuous positive airway pressure have shown promise in promoting healthy sleep patterns for neurodegenerative conditions [160,161]. A tailored light therapy protocol on sleep and cognition parameters in patients with AD of mild/moderate severity induced a circadian phase shift, improved sleep quality, and cognitive performance [162]. It is interesting to highlight that, also in the case of non-pharmacological options, their combination is highly promising. A regimen comprising a shortened light phase, time-restricted feeding, and exercise to strengthen the circadian system and enhance sleep quality in AD mice models demonstrated better cognitive performance but showed no effect on amyloid plaques or tau phosphorylation [163]. Nevertheless, several systematic reviews have found little or no robust evidence for these practices, suggesting the need for further high-quality research [164,165].

7. Conclusions and Perspectives

Here, we discuss the potential of polypharmacology, either by using drug combinations or MTDLs, for (i) addressing the challenges of delivering therapeutic agents across the BBB, and (ii) addressing CRD by modulating various components of the CC and related physiological processes. However, the exploitation of polypharmacology concepts in CRD is moving only glacially both at preclinical and clinical levels. Below, we reflect on the several challenges for both (i) and (ii) polypharmacology scenarios that may be important roadblocks for their routine applications:

(i) Polypharmacology as an efficient drug delivery option to improve BBB permeation
- Selectivity: Enhancing BBB permeability without allowing harmful substances to enter the brain is critical.
- Toxicity: The enhanced delivery of drugs and other substances through the BBB increases the risk of DDIs and unwanted side effects.
- Chronic Use: Long-term modulation of the BBB needs to be carefully monitored to avoid permanent damage.

(ii) Polypharmacology as an effective tool to modulate the complex molecular network of CRD in CNS diseases
- Balancing the activity: The CC is highly intricate, with many factors influencing it. A challenge in polypharmacology for CRD is achieving the right balance of targeting multiple components without causing unintended outcomes.
- Off-target effects: Polypharmacological tools may bind to undesired targets, disrupting normal CC functions, and can unintentionally affect interconnected biological networks, causing unpredictable effects.
- Long-term efficacy and safety: Long-term use of polypharmacological drugs that affect circadian rhythms must be monitored carefully for potential side effects, as circadian rhythms are involved in a wide range of physiological functions (e.g., metabolism, immune function, mood). Ensuring both safety and effectiveness over time is crucial, especially in chronic conditions.

In both (i) and (ii) scenarios, variations in individual circadian rhythms (due to chronotype, genetics, or existing health conditions) may require personalized approaches tailored to the individual's biological clock, genetic profile, and lifestyle. Hence, a deeper understanding of CRD molecular mechanisms will promote the application of polypharmacology for CC modulation. Polypharmacological CC modulators represent groundbreaking advancements, as they hold the potential to not only offer drug candidates for sleep disorders, as exemplified by dual orexin receptor antagonists, but also for other brain diseases. We are convinced that the future is bright for the field, so it is time for the development of polypharmacological interventions against circadian dysregulations in CNS diseases.

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.

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Data availability

No data was used for the research described in the article.

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