Published in final edited form as:

Pharmacol Ther. 2016 November ; 167: 108–119. doi:10.1016/j.pharmthera.2016.07.016.

Pharmacological targeting of the PDGF-CC signaling pathway for blood-brain barrier restoration in neurological disorders

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Abstract

Neurological disorders account for a majority of non-malignant disability in humans and are often associated with dysfunction of the blood-brain barrier (BBB). Recent evidence shows that despite apparent variation in the origin of neural damage, the central nervous system has a common injury response mechanism involving platelet-derived growth factor (PDGF)-CC activation in the neurovascular unit and subsequent dysfunction of BBB integrity. Inhibition of PDGF-CC signaling with imatinib in mice has been shown to prevent BBB dysfunction and have neuroprotective effects in acute damage conditions, including traumatic brain injury, seizures or stroke, as well as in neurodegenerative diseases that develop over time, including multiple sclerosis and amyotrophic lateral sclerosis. Stroke and traumatic injuries are major risk factors for age-associated neurodegenerative disorders and we speculate that restoring BBB properties through PDGF-CC inhibition might provide a common therapeutic opportunity for treatment of both acute and progressive neuropathology in humans. In this review we will summarize what is known about the role of PDGF-CC in neurovascular signaling events and the variety of seemingly different neuropathologies it is involved in. We will also discuss the pharmacological means of therapeutic interventions for anti-PDGF-CC therapy and ongoing clinical trials. In summary: inhibition of PDGF-CC signaling can be protective for immediate injury and decrease the long-term neurodegenerative consequences.

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Conflict of interest statement.

Drs. U. Eriksson, D.A. Lawrence, and L. Fredriksson are named inventors on a patent on modulating blood-neural barrier using PDGF-CC and PDGFR- α antagonist. The other author declares no potential conflict of interest.

Keywords

Blood-brain barrier; PDGF-CC; tPA; neurodegeneration; traumatic brain injury; stroke

1. Introduction

The 2013 Global Burden of Disease Study showed that cerebrovascular disease and traumatic injuries are among the top five leading causes for disability incidence worldwide regardless of country wealth (Murray et al., 2015) and are the main drivers for increase in years lived with disability (Vos et al., 2015). It is expected that the prevalence of cerebrovascular disabilities including stroke and neurodegenerative diseases will expand due to an aging population and lead to increased costs for direct and follow-up patient care (Olesen, Gustavsson, Svensson, Wittchen, & Jönsson, 2012). Apart from rare genetic predispositions or aging, which are the most evident risk factors for neurodegeneration, an additional aspect can contribute to increased prevalence of neurodegenerative diseases, namely early traumatic events and blood-brain barrier (BBB) dysfunction. It has become increasingly apparent that even mild cerebral traumatic events occurring early in life can lead to BBB dysfunction and thereby an increased risk for developing neurological disorders later in life (Gardner & Yaffe, 2015; Gardner et al., 2015; Johnson, Stewart, & Smith, 2012; Kang & Lin, 2012; Lunny, Fraser, & Knopp-Sihota, 2014; Seelen et al., 2014; Sivanandam & Thakur, 2012). Along with these epidemiological studies recent literature has identified various molecular signaling pathways governing BBB formation and maintenance. In this review we will focus on the platelet-derived growth factor (PDGF)-CC signaling pathway, since inhibition of this pathway has been shown to minimize BBB dysfunction in both acute and progressive experimental neuropathology models. We will first outline different neurological disorders and their link to BBB dysfunction, and then we will look into the mechanisms of PDGF-CC activation in light of its role in regulation of BBB integrity. Lastly, we will discuss the therapeutic attempts of PDGF-CC inhibition in neurological diseases with focus on future human therapy.

2. Neurological disorders, their association with BBB dysfunction and the role of PDGF-CC

a. Overview of neurological disorders and their association with BBB dysfunction

In order to improve treatments for acute neurologic conditions as well as neurodegenerative diseases it is necessary to understand the mechanisms leading to development of these disorders. A growing body of epidemiological evidence shows that apart from genetic predisposition and advanced age, previous incidence of cerebral trauma is associated with increased risk for neurodegeneration in the elderly.

For example: traumatic brain injury (TBI) with its clinical spectrum (Jordan, 2013) and acute pathophysiology (DeKosky, Blennow, Ikonomic, & Gandy, 2013; Prins, Greco, Alexander, & Giza, 2013) can have a wide array of long-term consequences. Initial head concussions induce chronic traumatic encephalopathy (CTE) with a broad pathology spectrum of amyloid, tau and TDP-43 inclusions together with behavioral and cognitive

impairment (McKee et al., 2013). The widespread accumulations of tau and amyloid pathology can persist for years after TBI (Johnson et al., 2012) together with sustained inflammation and white matter degeneration (Johnson et al., 2013). The long term consequences of TBI incidence leads to an increased risk for developing Alzheimer's disease (AD) (Sivanandam & Thakur, 2012), dementia (Johnson & Stewart, 2015), Parkinson's disease (PD) (Gardner et al., 2015) multiple sclerosis (MS) (Kang & Lin, 2012; Lunny et al., 2014) and amyotrophic lateral sclerosis (ALS) (Seelen et al., 2014). In a similar fashion, the incidence of stroke, along with its immediate cellular and clinical consequences (Moskowitz, Lo, & Iadecola, 2010; van der Worp & van Gijn, 2007), has also been shown to increase the risk for Alzheimer's disease (Thiel, Cechetto, Heiss, Hachinski, & Whitehead, 2014; Torre, 2013), ALS (Turner, Goldacre, Talbot, & Goldacre, 2015), vascular dementia (Allan et al., 2011; Savva & Stephan, 2010) and can lead to vascular forms of Parkinson's disease (Korczyn, 2015). This causal relationship between early trauma and neurodegeneration is not well understood. However, both the acute and the long-term degenerative events in the brain, despite their variety of origins, share common mechanisms of cerebral injury response that are associated with increased BBB dysfunction.

Blood vessels in the brain have unique properties with a tight barrier, known as the BBB separating circulating plasma contents from the brain parenchyma, and BBB dysfunction defined either as serum protein leak, hemorrhage or infiltration is thought to be a significant component leading to neurodegeneration (Obermeier, Daneman, & Ransohoff, 2013; Zlokovic, 2010). On the cellular level the formation of the physical barrier is manifested in the endothelial cells lining the vessel wall, with specialized tight junctions to prevent paracellular outflow, low vesicle transcytosis and active efflux mechanisms, which together minimize transcellular transport of plasma contents. These physical properties of the brain endothelium are induced and maintained by molecular signaling pathways (reviewed by (Chow & Gu, 2015)) as well as cellular interactions with perivascular cells including pericytes or smooth muscle cells (generally referred to as vascular mural cells) and astrocytes (reviewed by (Abbott, Rönnbäck, & Hansson, 2006; Obermeier et al., 2013; Zhao, Nelson, Betsholtz, & Zlokovic, 2015)).

Recent studies have increased our understanding of the contribution of the BBB to acute and long-term neuropathologies. For instance, an acute traumatic blow to the head or spinal cord results in an immediate BBB dysfunction (Sharma, 2011), followed by an increase in cerebral edema and inflammation (Donkin & Vink, 2010; Woodcock & Morganti-Kossmann, 2013). Therefore restoration of the BBB control is considered an important protective target for TBI therapy (Shlosberg, Benifla, Kaufer, & Friedman, 2010). Similarly, ischemic stroke results in an abrupt increased of BBB permeability followed by cerebral edema (Ayata & Ropper, 2002; Yang & Rosenberg, 2011) and in severe cases hemorrhagic transformation (Hom et al., 2010). The mechanisms leading to ischemic BBB opening are intensely studied for their therapeutic potential (Jin, Yang, & Li, 2010).

Apart from acute injury, BBB dysfunction is also reported in neurodegenerative diseases where a variety of contributing factors result in a slowly progressive disease course (Zlokovic, 2011). BBB dysfunction has also been described in patients with Alzheimer's disease (Sengillo et al., 2013; van Assema et al., 2012), ALS (Donnenfeld, Kascsak, &

Bartfeld, 1984; Miyazaki et al., 2011), MS (Gaitán et al., 2011) and PD (Bartels et al., 2008; Kortekaas et al., 2005). Among several pathways described to play a role in the development and maintenance of the BBB (reviewed by (Chow & Gu, 2015; Obermeier et al., 2013; Zhao et al., 2015)), in this review we will focus on the role of PDGF-CC in regulation of BBB integrity in various neuropathologies and the potential therapeutic applications of targeting this pathway.

b. PDGF-CC as a regulator of BBB integrity

PDGF-CC belongs to a family of secreted proteins which stimulate PDGF receptors α and β tyrosine kinase activity and have important roles in development and homeostasis of several tissues (reviewed by (Andrae, Gallini, & Betsholtz, 2008)). In the developing CNS the PDGFs have been shown to stimulate cell migration, formation of cranial neural crest and meningeal basement membranes (Andrae et al., 2016, 2008; Smith & Tallquist, 2010). In the adult CNS PDGF-CC, and later also PDGF-BB, have been shown to affect BBB permeability, the latter through maintenance of the pericyte-endothelial interaction (Armulik et al., 2010; Armulik, Genové, & Betsholtz, 2011; Su et al., 2008). Intraventricular injection of active PDGF-CC protein is sufficient to induce BBB opening (Su et al., 2008) and its inhibition was shown to reduce BBB dysfunction and indicated promising therapeutic effects in experimental ischemic stroke (Su et al., 2008), spinal cord injury (Abrams et al., 2012; Sharp, Yee, & Steward, 2014), TBI (Su et al., 2015), MS (Adzemovic, Zeitelhofer, Eriksson, Olsson, & Nilsson, 2013), seizures (Fredriksson et al., 2015) and ALS neurodegeneration (Lewandowski et al., 2016). In order to better understand the role of PDGF-CC in regulation of BBB integrity we will first describe its structure and mechanisms of activation.

3. PDGF-CC structure and mechanisms of activation

a. Gene and protein organization

PDGF-CC was discovered as a novel member of the PDGF family (Li et al., 2000) about three decades after the identification of PDGF-AA and PDGF-BB (Johnsson, Heldin, Westermark, & Wasteson, 1982; Kohler & Lipton, 1974). The PDGF family has since been extended with an additional member, PDGF-DD (Bergsten et al., 2001; LaRochelle et al., 2001). All the members of this family contain a structurally well-conserved growth factor domain (GFD), that is characterized by a pattern of eight invariant cysteine residues (reviewed in (Fredriksson, Li, & Eriksson, 2004)) (Figure 1). PDGF-CC and PDGF-DD contain an additional unique structural module, referred to as the CUB (Complement subcomponents C1r/C1s, Urchin EGF-like protein, Bone morphogenic protein-1) domain (Bergsten et al., 2001; Li et al., 2000) (Figure 1).

The human *PDGFC* gene is located on chromosome 4 and spans around 200 Kb of genomic DNA (Uutela et al., 2001). It consists of 6 coding exons, where exon 1 encodes the signal peptide, and exons 2 and 3 encode the structural CUB domain. Exon 4 in the *PDGFC* gene encodes the region between the two structural domains in the growth factor and exons 5 and 6 encode the GFD (Uutela et al., 2001). *PDGFC* promoter is similar to the one of *PDGFA* with binding sites for Sp1, Egr-1, Smad and Ets transcription factors and was shown to be stimulated by binding of Sp1 and Egr-1 proteins (Midgley & Khachigian, 2004; Sanchez-

Guerrero, Midgley, & Khachigian, 2008) (Figure 2). The Egr-1 and Sp1 binding sites at –986 contain a C>T single nucleotide polymorphism termed rs28999109. It abolishes the Sp1 and Egr-1 consensus binding motif and was shown to decrease promoter activity (Choi et al., 2009) (Figure 2). The mRNA encoding PDGF-C results in a polypeptide chain of 345 amino acids in length that assembles into a PDGF-CC, homodimer through intra- and inter-chain disulphide bond formation between cysteine residues in the GFD (Figure 1). The GFD is around 100 amino acid residues in length and crystallization studies have shown that the two GFD in a PDGF-BB homodimer are arranged in an anti-parallel fashion (Oefner, D'Arcy, Winkler, Eggimann, & Hosang, 1992). Given the close similarities in the primary structures of the GFD in all the PDGFs, it is likely that also PDGF-CC adopts this anti-parallel organization during dimerization. The N-terminal of the GFD in the PDGF-C polypeptide chain is the CUB domain (Li et al., 2000). It is now widely accepted that one of the most important roles of the CUB domains in PDGF-CC is to prevent the full-length dimer from binding its cognate receptor by sterically blocking the receptor binding surfaces in the GFDs. PDGF-CC is thus secreted as a latent factor requiring proteolytic removal of the CUB domains to release the GFD dimer, which can subsequently bind and activate the tyrosine kinase receptor PDGFR- α (discussed in further detail below) to regulate various biological processes. Another important function of the CUB domains in PDGF-CC is that they play a role in the binding and positioning of its activating enzyme, tissue-type plasminogen activator (tPA) (Fredriksson, Ehnman, Fieber, & Eriksson, 2005; Fredriksson, Li, Fieber, Li, & Eriksson, 2004). CUB domains have been found in many different proteins where they are involved in protein – protein or protein – carbohydrate interactions (Bork & Beckmann, 1993) and it has therefore been proposed that the CUB domains in PDGF-CC, as well as PDGF-DD, might confer retention of the growth factors to the pericellular space after secretion (Li et al., 2000). Studies on PDGF-DD do not however support this hypothesis, instead these studies suggest that the CUB domain in PDGF-CC and PDGF-DD conceals a retention motif that is exposed by proteolytic cleavage (Ehnman, Li, Fredriksson, Pietras, & Eriksson, 2009). Recent results have suggested that the CUB domain in PDGF-CC transduce intracellular signaling events independently of the GFD (Hayes et al., 2015). This notion is not supported by studies in mice where overexpression of only the GFD in heart induced cardiac fibrosis, hypertrophy, and dilated cardiomyopathy whereas overexpression of only the CUB domain did not (Pontén et al., 2003).

b. Mechanisms of activation

PDGF-CC is secreted as an inactive dimer of about 90 kDa in size that requires extracellular proteolytic activation through removal of its N-terminal CUB domains (Li et al., 2000) (Figure 2). We have previously shown that the trypsin-like serine protease, tissue-plasminogen activator (tPA), is a potent activating enzyme of PDGF-CC (Fredriksson, Li, Fieber, et al., 2004) and this has later been confirmed by others (Hurst, Najy, Ustach, Movilla, & Kim, 2012; Lei et al., 2007; Riehle et al., 2014). It has since been shown that PDGF-DD is activated through proteolysis by the other known plasminogen activator, urokinase plasminogen activator (uPA) (Ehnman et al., 2009; Ustach & Kim, 2005). Cleavage of latent PDGF-CC by tPA releases a GFD dimer of approximately 35 kDa in size (Fredriksson, Li, Fieber, et al., 2004) (Figure 2). Identification of tPA as the catalytic enzyme responsible for PDGF-CC activation was rather unexpected because tPA is best

known for its role in fibrinolysis within blood plasma, where it cleaves and activates plasminogen into the broad specificity protease plasmin (Collen, 2001). However, especially in the CNS it is evident that tPA regulates biologic processes that are not mediated through activation of plasminogen, but through other downstream substrates (Lemarchant et al., 2012; Su, Fredriksson, Schielke, Eriksson, & Lawrence, 2009).

Based on alignments with the other PDGFs, a highly conserved tribasic sequence has been identified in all four chains (Bergsten et al., 2001; Li et al., 2000). These sites are located approximately 15 amino acids upstream of the first cysteine residue of the GFDs. In human PDGF-C this tribasic sequence is RKSR²³⁴ and using mutagenesis of this tribasic sequence in PDGF-CC it was confirmed that tPA-mediated cleavage depends on this site with R²³¹ in human PDGF-CC being of particular importance (Fredriksson et al., 2005) (Figure 1). The cleavage site of human PDGF-C was predicted to be at R²³⁴-V²³⁵ and observed at K²²⁵-A²²⁶ (Gilbertson et al., 2001). Cleavage of PDGF-AA and PDGF-BB is also known to occur at these sites, whereas cleavage of PDGF-DD has been reported to occur further away from the GFD (LaRochelle et al., 2001; Ustach & Kim, 2005). tPA-mediated cleavage of PDGF-CC depends not only on this conserved tribasic site, but also on correct positioning of tPA through interaction between the second kringle domain of the protease with both the CUB and GF domains in PDGF-CC (Fredriksson et al., 2005; Fredriksson, Li, Fieber, et al., 2004).

Apart from tPA, other proteases, including plasmin, uPA and matriptase have been shown to cleave PDGF-CC in *in vitro* biochemical assays and experimental disease models (Lei, Velez, Hovland, Hirose, & Kazlauskas, 2008; Li et al., 2000; Riehle et al., 2014). The existence of additional PDGF-CC activating proteases is supported by recent findings illustrating an incomplete phenotypic overlap in the brains of PDGF-CC and tPA deficient mice, but it is considered unlikely that broad specificity proteases, such as plasmin and matriptase will be relevant for regulation of PDGF-CC activity in adult physiology (Fredriksson, Li, Fieber, et al., 2004). Nevertheless it is certainly plausible that these proteases might play a role in activation of the growth factor during developmental processes and in pathologic conditions, although this requires further investigation.

The activation of PDGF-CC by tPA is regulated by a number of *serine* *protease* *inhibitors* (serpins) and co-receptors (Su et al., 2009). Outside the CNS, tPA activity is mainly regulated through inhibition by plasminogen activator inhibitor-1 (PAI-1, *SERPINE1*) (Collen, 2001), whereas in the CNS, neuroserpin (*SERPINI1*) was recently found to be the physiologic relevant inhibitor of tPA activity (Fredriksson et al., 2015) (Figure 2). In the CNS, where tPA is believed to exert unique functions distinct from its role in fibrinolysis, we have previously demonstrated that activation of PDGF-CC in the neurovascular interface requires existence of LDL receptor-related protein (LRP1) (Su et al., 2008) (Figure 2). Thus, we speculate that efficient activation of PDGF-CC in the neurovascular unit and subsequent signaling through PDGFR- α depends on the formation of a multimeric protein complex involving, at least, tPA, PDGF-CC, LRP1, and PDGFR- α (Su et al., 2009) (Figure 2).

c. Protein function in physiology and disease

PDGF signaling regulates diverse cellular functions in connective tissue cells, such as fibroblasts and smooth muscle cells, as well as in cells of neuroectodermal origin (Andrae et al., 2008). PDGF signaling is important for recruitment of supportive cells during the process of angiogenesis, but these molecules also display other functions. It is now widely appreciated that the PDGF ligand – receptor systems are essential for embryogenesis (Betsholtz, 2004), and that overactivity of PDGF signaling correlates to several pathological conditions (Heldin, 2013, 2014). This has led to investigations targeting the PDGF pathway in various diseases including pulmonary arterial hypertension (Antoniou, 2012), cancers (Östman, 2004) as well as fibrotic diseases and systemic sclerosis (Trojanowska, 2008). Considering that PDGF-CC signaling has also been linked to both fibrotic disease (Eitner et al., 2008) and breast cancer (Hurst et al., 2012) it is possible that targeting this growth factor in these non-neurologic disorders by the means summarized below may be an effective therapeutic approach, however, this review is focused only on the potential indications in the CNS.

Deletion of the genes encoding the various PDGF ligands and receptors has provided invaluable information about their biological function during embryonic development. Deletion of *Pdgfra* gene (Soriano, 1997), encoding the receptor for PDGF-AA and PDGF-CC, results in a phenotype that is similar, but more severe than elimination of either *Pdgfa* (Boström et al., 1996) or *Pdgfc* (Ding et al., 2004) alone. Combined deletion of *Pdgfa* and *Pdgfc* was found to recapitulate the *Pdgfra* knockout phenotype and indicates that PDGF-AA and PDGF-CC are the primary PDGFR- α ligands, at least during embryonic development (Ding et al., 2004). Our previous data showed that *Pdgfc* deficiency on a C57BL/6 background is largely compatible with postnatal life and offered the opportunity to study physiologic and pathophysiologic roles of this protein in the adult mouse (Fredriksson et al., 2012). These *Pdgfc* deficient mice have numerous congenital defects, including cerebral ventricular malformations with a distorted ependymal lining and abnormal cerebral vascularization, features that we recently found also to be associated with ablation of tPA (Stefanitsch, Lawrence, Olverling, Nilsson, & Fredriksson, 2015), which suggests a direct *in vivo* link between PDGF-CC signaling and its activating enzyme in CNS development. More recently, studies utilizing *Pdgfc* deficient mice with reduced *Pdgfra* levels have identified additional roles for PDGF-CC in CNS development including abnormal meningeal formation and neuronal over-migration in the cerebral cortex (Andrae et al., 2016). *Pdgfc* deletion studies in mice have also revealed that PDGF-CC is essential for palatogenesis (Ding et al., 2004). Interestingly, human linkage and association studies show strong linkage between a 30-cM region on human chromosome 4, where the *PDGFC* gene maps, or the rs28999109 polymorphism in the *PDGFC* promoter and occurrence of cleft lip with or without cleft palate (CL/P) (Choi et al., 2009; Marazita et al., 2002), which suggests that PDGF-CC has role in palatogenesis also in humans (Calcia et al., 2013; Choi et al., 2009; Jugessur et al., 2009).

The role of PDGF-CC in adult physiology is much less well understood. tPA was identified as the activating enzyme of PDGF-CC (Fredriksson, Li, Fieber, et al., 2004) and has been demonstrated to be both necessary and sufficient to increase vascular permeability in the

brain through control of BBB integrity (Yepes et al., 2003). This tPA-induced opening of the BBB was reported to depend on binding of tPA to the cell surface receptor LRP1 and cleavage of an unknown substrate (Yepes et al., 2003) which was later shown to be PDGF-CC (Su et al., 2008). In addition, LRP1 had been shown to act as a co-receptor of PDGF receptor signaling in an experimental model of atherosclerosis (Boucher, 2003; Loukinova et al., 2002). Based on these findings it was hypothesized that PDGF-CC might be a downstream substrate of tPA within the neurovascular unit involved in regulation of BBB integrity, which was later confirmed by studies in mice (Su et al., 2008). It was demonstrated that both tPA and LRP1 act upstream of active PDGF-CC in controlling cerebrovascular permeability and that LRP1 expressed on the cell surface enhances tPA-mediated activation of latent PDGF-CC and subsequently PDGFR- α signaling expressed on perivascular astrocytes (Su et al., 2008). Activation of PDGFR upon ligand binding depends on receptor dimerization and subsequent phosphorylation of the intracellular domains (reviewed by (Heldin, Östman, & Rönstrand, 1998)). Activated PDGFR- α and - β receptors stimulate several downstream signaling pathways including Ras and p38 MAPK (mitogen-activated protein kinase), PI3K and Plc- γ (reviewed by (Andrae et al., 2008; Demoulin & Essagher, 2014)) (Figure 2) with the p38 MAPK activity being particularly relevant for BBB integrity upon PDGFR- α stimulation (Ma et al., 2011).

4. tPA – PDGF-CC – PDGFR- α signaling axis in the CNS: Means of pharmacological inhibition and therapeutic applications

a. Means of pharmacological PDGF-CC inhibition

As mentioned above PDGF-CC exerts its function by binding to and activating PDGFR- α signaling. Signal transduction can be inhibited by several approaches (reviewed by (Andrae et al., 2008)) and here we will review the means reported to inhibit PDGF-CC – PDGFR- α signaling that are applicable for translational therapies. First, the ligand can be blocked from interacting with the receptor by antibodies targeting the PDGF-CC protein. This has been illustrated in a number of studies using anti-PDGF-CC neutralizing antibodies in models of experimental stroke (Su et al., 2008), fibrosis (Eitner et al., 2008; Martin et al., 2013) and macrophage survival (Son, Na, Hwang, & Seok, 2014) (Figure 3 and Table 1). Second, antibodies directed against the ligand-binding domain of the PDGFR- α have been successfully used to inhibit receptor activation in myocardial infarction (Zymek et al., 2006) and cancer (Dolloff, Russell, Loizos, & Fatatis, 2007; Laing et al., 2013; Loizos et al., 2005; Taniguchi et al., 2008) (Figure 3 and Table 1). Antibody-based methods have the advantage of target specificity and low toxicity, but may be costly to manufacture. Lastly, small molecules, including tyrosine kinase inhibitors (TKIs), can be used to prevent the intracellular domain phosphorylation of PDGFR- α after ligand binding. Tyrosine kinase inhibitors with high specificity for PDGFR- α/β inhibition and IC₅₀ values below 400 nM include imatinib, sunitinib, sorafenib, pazopanib and nilotinib (Heldin, 2013, 2014) (Figure 3 and Table 1). These inhibitors all have other targets beyond PDGFR and inhibit several other tyrosine kinases. Indeed, most were developed as inhibitors of other target receptors. For example imatinib was developed as an inhibitor of Abl kinase and sunitinib can also inhibit VEGFR. Numerous other TKIs have been designed to inhibit PDGFRs and are currently tested by pharmaceutical companies for therapeutic applications (Dai, 2010). For

broader description on TKI selectivity and sensitivity see the reviews by (Heldin, 2014; Levitzki, 2013). The advantage of TKIs is that they have been approved by the FDA for the treatment of human diseases and can provide options for faster translation into therapy. Taking into consideration the rather broad receptor selectivity of many of the TKIs development of antibodies for specific PDGF ligands and receptors could become a more precise therapeutic approach with minimal off-target effects. Below we will describe the neurological conditions where inhibition of the PDGF-CC signaling has been associated with restoration of the BBB with beneficial neuroprotective effects.

i. Inhibition of PDGF-CC signaling restores BBB and is neuroprotective in acute trauma—The discovery that PDGF-CC can induce BBB permeability was made when it was shown that injection of active PDGF-CC or tPA proteins into the cerebral spinal fluid (CSF) of the cerebral ventricles of mice was sufficient to cause increased BBB permeability (Su et al., 2008; Yepes et al., 2003). Further, tPA-induced BBB opening could be decreased with neutralizing anti-PDGF-CC antibodies, indicating that PDGF-CC acts downstream of tPA in the CNS. BBB dysfunction during cerebral ischemia was also associated with increased phosphorylation of PDGFR- α and provided the mechanistic outline for a tPA – PDGF-CC – PDGFR- α signaling axis *in vivo*. The effect of BBB disruption after intracerebral injection of PDGF-CC was confirmed in another study (Jackman et al., 2013), which showed that genetic deficiency for progranulin (*Pgrn*) exacerbates the degree of PDGF-CC – induced BBB dysfunction. Together, these findings provide a novel concept to pharmacologically target the PDGF-CC pathway to prevent or reduce BBB dysfunction in a range of neurological disorders.

Ischemic stroke results in a rapid increase in perivascular tPA activity and increased BBB permeability (Yang & Rosenberg, 2011; Yepes et al., 2003). Restoration of BBB integrity is therefore considered an important therapeutic target (Jin et al., 2010). Our group showed that inhibition of the PDGF-CC signaling by neutralizing antibodies or imatinib reduced BBB dysfunction in stroke and that this was associated with significantly reduced infarct size and hemorrhage (Su et al., 2008). These findings were confirmed by independent studies in mice (Ma et al., 2011) or rats (Zhan et al., 2015) which showed that inhibition of PDGFR- α with imatinib reduced BBB opening and cerebral edema. It was demonstrated that p38 MAPK is responsible for PDGFR- α mediated increase in BBB permeability since the use of SB203580, a p38 MAPK inhibitor, decreased BBB dysfunction induced by a PDGFR- α ligand (Ma et al., 2011). Another study also supported the role of PDGF-CC in hemorrhagic transformation in humans by showing that increased levels of PDGF-CC in the plasma of stroke patients were associated with increased hemorrhagic transformation after thrombolytic therapy with tPA (Rodríguez-González et al., 2013). These findings reinforced the concept that the BBB may be a therapeutic target in the treatment of stroke, and opened up the possibility that similar protective effects of PDGF-CC pathway inhibition could also be present in other forms of traumatic injuries to the CNS.

The early manifestation of seizures is associated with BBB dysfunction (Marchi, Granata, Ghosh, & Janigro, 2012) and is suggested to be sufficient to induce onset (Marchi et al., 2007) or contribute to progression to epilepsy (van Vliet et al., 2007). Intriguingly, tPA was one of the first genes recognized to be activated early after seizure onset (Qian, Gilbert,

Colicos, Kandel, & Kuhl, 1993) and its genetic deficiency raises threshold for seizure induction (Tsirka, Gualandris, Amaral, & Strickland, 1995; Yepes et al., 2002). In a recent study we showed that imatinib delays onset and progression of kainate-induced seizures in wild-type mice but not in tPA knockout mice (Fredriksson et al., 2015). This lack of cumulative protection of imatinib treatment in tPA knockout mice suggests that during seizures both tPA and PDGFR- α operate in the same signaling pathway. Moreover, inducible deletion of recombinant PDGFR- α in perivascular astrocytes reduced progression of convulsive seizure behavior (Fredriksson et al., 2015) and indicates that intervention at the PDGFR- α , acting downstream of tPA, is sufficient to have an effect on seizure progression.

Traumatic spinal cord injuries also lead to abrupt BBB dysfunction (Sharma, 2011) and restoration of barrier integrity is considered a vital therapeutic target (Shlosberg et al., 2010). Our group has shown that rats with spinal cord injury treated with imatinib display improved BBB function, and reduced formation of glial scar and deposition of chondroitin sulphate proteoglycans (Abrams et al., 2012). These beneficial effects on histological preservation resulted in an improved locomotor and sensorimotor hindlimb performance together with preserved bladder function after injury (Abrams et al., 2012). Our findings were confirmed by an independent re-assessment study where imatinib treatment preserved bladder function (Sharp et al., 2014). In a similar fashion imatinib treatment was proven to improve outcome of traumatic brain injury in a mouse model (Su et al., 2015). Imatinib delivered 45 min after TBI reduced BBB dysfunction, edema, lesion size, and was able to preserve cognitive function in the Morris water maze. Importantly, patients with TBI had increased concentrations of PDGF-CC in the CSF in proportion with increased severity of trauma, which would suggest its use as a marker for neurological injury.

ii. Inhibition of PDGF-CC signaling restores BBB properties and is neuroprotective in neurodegenerative disorders—Apart from targeting the PDGF-CC pathway to restore BBB in acute cerebral trauma, such therapeutic approach might also be applied to other neuropathologies where CNS injury develops over time. For example imatinib treatment of rats with experimental autoimmune encephalopathy (EAE) model of MS reduced BBB dysfunction, CNS inflammation and demyelination (Adzemovic et al., 2013). In this study imatinib was also able to suppress the peripheral immune response and reduce T-cell recruitment, which together with BBB restoration significantly improved EAE scores. Another example of PDGF-CC pathway activation during long-term neurodegeneration was found in ALS. ALS is a progressive neurodegenerative disorder associated with aging and, apart from mutations causing the familial forms of the disease, factors triggering sporadic ALS are poorly understood. Dysfunction of the BBB was proposed as one of the elements contributing to onset of ALS since it was described in humans (Halliday et al., 2015; Leonardi, Abbruzzese, Arata, Cocito, & Vische, 1984; Miyazaki et al., 2011) and was present before the onset of disease in transgenic ALS mice models (Miyazaki et al., 2011; Zhong et al., 2008). We have shown that presymptomatic activation of the PDGF-CC pathway leads to dysfunction of the BBB and accelerates onset of ALS neurodegeneration (Lewandowski et al., 2016). Spinal cords of presymptomatic *SOD1^{G93A}* ALS mice had increased expression and activity of tPA together with

accumulation of cleaved PDGF-CC protein. Moreover, the presymptomatic mice showed signs of BBB dysfunction on vessels surrounded with PDGFR- α positive astrocytes with overall increase of PDGFR- α phosphorylation in tissue lysates (Lewandowski et al., 2016). We found similar evidence for activation of the PDGF-CC pathway in motor neurons from spinal cords of sporadic ALS patients where gene expression of both *PDGFC* and *PLAT* was increased. Inhibition of the PDGF-CC pathway by either imatinib treatment or crossing with *Pdgfc* null strain restored BBB properties, reduced neuron degeneration and delayed the onset of disease in *SOD1^{G93A}* ALS mice. The effect of *Pdgfc* ablation on onset was the strongest reported delay for a genetic intervention in this strain and outperformed transgenic overexpression of parvalbumin (Beers et al., 2001) or glutamate transporter EAAT2 (Guo et al., 2003). Analogous result was found in neuronal cells from sporadic ALS patients where decreased expression of *PDGFC* or *PLAT* was significantly correlated with increased latency to disease onset (Lewandowski et al., 2016). The beneficial effects of BBB restoration in EAE and ALS suggest that, apart from immediate injury response, inhibition of PDGF-CC can be applied as a therapy of neurodegenerative diseases that develop over time. Moreover, activation of the PDGF-CC pathway and subsequent BBB dysfunction can provide a mechanistic explanation for increased risk of neurodegenerative diseases after traumatic events.

b. PDGF-CC as a common target for preventive treatments in neurodegeneration

The similarity of PDGF-CC induced BBB dysfunction in both acute and long-term neurological conditions provides a plausible mechanistic explanation why cerebral trauma leads to increased risk for neurodegeneration in later life and can be outlined in three steps. First, initial injury by direct trauma, ischemia or seizures results in early expression and increase of tPA activity from neurons (Qian et al., 1993; Sashindranath et al., 2012; Yepes et al., 2003). Second, as a consequence of tPA activity in stroke or trauma, PDGF-CC becomes cleaved producing an active ligand and its levels in the CSF correspond to the degree of cerebral injury (Su et al., 2008, 2015). Third, PDGF-CC mediated PDGFR- α activation is sufficient to induce BBB opening known to exacerbate the downstream consequences of injury like cerebral edema, neuroinflammation and neuronal death (Ma et al., 2011; Su et al., 2008) (Figure 4). As a consequence PDGF-CC induced BBB dysfunction, apart from the immediate consequences, can provide additional stressor stimuli for long-term neurological conditions where cumulative effects of environmental factors and genetic predisposition can lead to sporadic neurodegeneration.

The concept of BBB dysfunction as contributor to a spectrum of cerebral diseases is supported by epidemiological evidence where previous history of trauma or stroke increases the risk for various forms of neurodegeneration (Gardner & Yaffe, 2015; Perry et al., 2016). In particular, prior incidence of trauma was shown to increase the risk of ALS in the general population up to 11 fold (Chen, Richard, Sandler, Umbach, & Kamel, 2007; Pupillo et al., 2012; Seelen et al., 2014; Turner et al., 2015) and is thought to be the cause for the high ALS incidence in military servicemen (Beard et al., 2016; Horner et al., 2003; Seals, Kioumourtzoglou, Gredal, Hansen, & Weisskopf, 2015; Weisskopf et al., 2005) and professional football players (Chio, 2005; Lehman, Hein, Baron, & Gersic, 2012). Similarly, the principle of PDGF-CC induced BBB opening might hypothetically also be in play in

other neurodegenerative disorders including AD, PD or vascular dementia where BBB dysfunction and a history of trauma has been described as a risk factor (Allan et al., 2011; Gardner et al., 2015; Johnson & Stewart, 2015; Korczyn, 2015; Savva & Stephan, 2010; Sivanandam & Thakur, 2012; Thiel et al., 2014; Torre, 2013).

BBB restoration was most extensively studied with the use of imatinib as a means to inhibit the PDGFR- α signaling. Other TKIs like sunitinib, nilotinib, dasatinib and masitinib have similar potency to inhibit the PDGFR- α and have been used in numerous preclinical studies in the context of neurodegeneration. We will now describe several instances where the beneficial effects of these TKIs on neurodegeneration are likely to have an effect on inhibition of the PDGFR- α and restoration of the BBB. For instance: sunitinib was reported to reduce hypoxia-induced cerebral dysfunction and edema formation in rat brains (Sarawat, Nehra, Chaudhary, & Prasad, 2015). Nilotinib was reported to reduce neurodegeneration in a TDP-43 induced model of ALS (Wenqiang et al., 2014), AD model (Lonskaya, Hebron, Selby, Turner, & Moussa, 2015), as well as MPTP-induced and α -synuclein overexpression PD models (Hebron, Lonskaya, & Moussa, 2013; Karuppagounder et al., 2014). Dasatinib was shown to improve memory and decrease microglia activation in AD mice (Dhawan & Combs, 2012), to delay the onset of EAE (Azizi, Goudarzvand, Afraei, Sedaghat, & Mirshafiey, 2015), and to extend survival and motor skills in *SOD1^{G93A}* ALS mice (Katsumata et al., 2012). Masitinib was shown to be neuroprotective in ischemic stroke (Kocic et al., 2015) and is being tested in a clinical trial for MS, ALS and AD (Piette et al., 2011; Salvado et al., 2015; Vermersch et al., 2012). These studies assume other protein kinases as primary targets for TKIs and do not analyze BBB as a measurable outcome, yet it is probable that observed beneficial effects include or are partly due to restoration of the BBB. The beneficial effects of these TKIs warrant future studies where BBB restoration should be a measurable outcome and allow us to better understand their therapeutic potential.

5. Conclusions and future perspectives

Despite increasing knowledge of the cellular and molecular mechanisms driving the cerebral injury response the therapeutic options for treatment of both acute and progressive forms of neuropathology remain limited. Ischemic injury and TBI still remain the leading causes for premature death and long-term disability with high costs of secondary care (Ma, Chan, & Carruthers, 2014; Olesen et al., 2012; Vos et al., 2015). At the same time reliable standardized diagnosis, particularly for mild traumatic events, is scarce and as a result they are underrepresented in the epidemiological statistics for associations with long-term consequences of cerebral injury (Roozenbeek, Maas, & Menon, 2013; Rusnak, 2013). Therefore development of reliable methods for diagnosis of acute BBB dysfunction is needed to fully appreciate its long-term effects. For example, improved imaging techniques (Mac Donald et al., 2011; Weissberg et al., 2014) together with novel CSF protein biomarkers of injury (DeKosky et al., 2013; Zetterberg, Smith, & Blennow, 2013) could lead to increased awareness of BBB changes in cerebral injury. The presence of PDGF-CC in CSF after trauma could likely become a very informative biomarker since its direct consequences on BBB dysfunction are now more evident. More importantly, PDGF-CC signaling can become a common therapeutic target to prevent BBB dysfunction and improve

recovery in for both acute forms of injury seen in TBI, stroke or seizures as well as in progressive neurodegenerative disorders such as MS and ALS. Imatinib is an FDA-approved drug, and can be administered orally as a tablet, it is most suitable for short-term treatments in acute forms of cerebral injury and is currently evaluated in clinical trial for ischemic stroke (EudraCT Number: 2010-019014-25). However, due to its effects on other signaling pathways, development of antibody-based therapeutic strategies might be more suitable for long term treatments of progressive neurodegenerative disorders.

Acknowledgments

The authors would like to thank Dr. Robert Schnell for generation of protein structure images used in Figure 1. The structural models of PDGF-C CUB domain were based on human Cubulin (residues 935–1042, PDB: 3KQ4, (Andersen, Madsen, Storm, Moestrup, & Andersen, 2010)) and GF domain was based on human PDGF-A (residues 118–179, PDB: 3MJK, (Hye-Ryong Shim et al., 2010)) obtained from the MODBASE database (Haas et al., 2013). The authors would like to acknowledge grant support from the following agencies: the Björklunds fund by the Swedish Society of Medicine SLS-499431 (S.A.L.), the Swedish Agency for Innovation Systems, Grant 2011-03503 (LF), the Swedish Research Council, Grant 2012-1853 (LF), the National Institutes of Health Grants HL055374 and NS079639 (DAL), the Swedish Heart and Lung Foundation 2012-0077 (U.E.), the Swedish Cancer Foundation 2014/630 (U.E.), and the Swedish Research Council 2011-3861 (U.E.).

Abbreviations

BBB	Blood-brain barrier
PDGF	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptor
CNS	Central nervous system
tPA	Tissue plasminogen activator
AD	Alzheimer's disease
PD	Parkinson's disease
ALS	Multiple sclerosis (MS), Amyotrophic lateral sclerosis
TBI	Traumatic brain injury
CTE	chronic traumatic encephalopathy
TKI	Tyrosine kinase inhibitor
CSF	cerebral spinal fluid
EAE	Experimental autoimmune encephalopathy
GFD	Growth factor domain
CUB	<u>C</u> omplement subcomponents C1r/C1s, <u>U</u> rchin EGF-like protein, <u>B</u> one morphogenic protein-1
LRP	LDL receptor-related protein

CL/P	Cleft lip with or without cleft palate
MAPK	Mitogen-activated protein kinase

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Figure 1. Putative structure of the human PDGF-C CUB and GF domains

Domains are modeled separately and based on homology with human Cubulin (PDB: 3KQ4, residues 935–1042) and human PDGF-A (PDB: 3MJK, residues 118–179). The segment between CUB and GF domains is represented as dots with displayed cleavage site sequence. RV – predicted and KA – observed cleavage site (Gilbertson et al. JBC 2001), RKSR – putative processing site (Li et al. Nat. Cell. Biol. 2000), R²³¹ – essential for tPA-mediated cleavage (Fredriksson et al. JBC 2005). The disulfide bond-forming cysteines conserved in the GF domain of PDGF family are highlighted in orange.

Figure 2. Overview of PDGF-CC signaling

PDGFC gene expression in the signaling cell is regulated by the Egr-1 and Sp1 transcription factors. SNP rs28999109 disrupts the consensus binding sequence for Egr-1 and Sp1 and decreases promoter activity. In the extracellular matrix the inactive PDGF-CC homodimer containing the CUB domains is cleaved by tPA to release the active dimer consisting of growth factor domains. PAI-1 is a tPA inhibitor in plasma fibrinolysis and Neuroserpin inhibits tPA in the CNS parenchyma. PDGFR- α is present in the responding cell together with LRP1, a co-receptor that facilitates efficient cleavage of PDGF-CC by tPA. Upon ligand binding PDGFR- α becomes phosphorylated and activates intracellular signaling pathways including PI3K, Ras MAPK, p38 MAPK and Plc- γ .

Figure 3. Pharmacological means of inhibition for PDGF-CC – PDGFR- α signaling

Active PDGF-CC ligand can be targeted with neutralising antibodies to prevent receptor binding. In similar fashion ligand binding to PDGFR- α can be prevented by antibodies targeting the extracellular domains of the receptor. PDGFR- α intracellular domain phosphorylation and signaling activity can be reduced with tyrosine kinase inhibitors. For detailed description see Table 1.

Figure 4. Schematic diagram of the neurovascular unit in CNS injury

(I) Expression of tPA protein is induced and it becomes available in the extracellular matrix.

(II) Proteolytically active tPA cleaves off the regulatory CUB domain (white) from the PDGF-CC dimer core (blue) yielding an active ligand.

(III) Activation of PDGFR-α located on the perivascular astrocytes exerts influence on endothelial uptake by an unknown mechanism (IV) and results in BBB deregulation (V).

Table 1

Pharmacological methods of targeting the PDGF-CC – PDGFR- α pathway.

	Means of inhibition	Effect on PDGF-CC pathway	Other biological functions tested	Effect on BBB	Effect on neurodegeneration	Reference
Anti PDGFR- α antibodies	PDGF-CC 615 Ab (rabbit)	Inhibits PDGF-CC induced PDGFR- α phosphorylation	Inhibits TPA induced BBB dysfunction	Reduces tPA induced BBB dysfunction	- N.S. (not studied)	(Su et al., 2008)
	PDGF-CC Ab (sheep)	Inhibits PDGFC induced PDGFR- α phosphorylation	Reduction of kidney fibrosis and myofibroblast accumulation.	- N.S.	- N.S.	(Eitner et al., 2008) (Martin et al., 2013)
	PDGF-CC HH1-57 Ab (mouse)	Inhibits PDGF-CC induced PDGFR- α phosphorylation	PDGFR- α activation assay in vitro	- N.S.	- N.S.	(Lei et al., 2009)
Anti PDGFR- α antibodies	PDGFR- α 3G3 Ab (mouse)	Blocks ligand binding to PDGFR- α .	Inhibits growth of glioblastoma leiomyosarcoma xenografts	- N.S.	- N.S.	(Loizos et al., 2005)
			Inhibits Akt pathway in prostate cancer cells	- N.S.	- N.S.	(Dolloff et al., 2007)
			Decreased proliferation and survival of hepatoma cell lines	- N.S.	- N.S.	(Stock et al., 2007)
			Reduced growth of ovarian cancer	- N.S.	- N.S.	(Matei et al., 2005)
	PDGFR- α MEDI-575 Ab (mouse)	Blocks ligand binding to PDGFR- α .	Reduced stromal fibroblast content and signaling in non-small cell lung cancer xenografts.	- N.S.	- N.S.	(Laing et al., 2013)
	PDGFR- α AF1062 Ab (goat)		Reduced rhabdomyosarcoma progression in vivo. Prevented ciliary instability in PDGF-AA stimulated MEFs.	- N.S.	- N.S.	(Taniguchi et al., 2008) (Jacoby et al., 2009)
PDGFR- α tyrosine kinase inhibitors	PDGFR- α α R1 Ab (mouse)	Blocks ligand binding to PDGFR- α .	Inhibits PDGF-AA mediated growth of hematopoietic cells.	- N.S.	- N.S.	(LaRoche et al., 1993) (Yu, Mahadevan, LaRoche et al., Pierce, & Heidaran, 1994)
	Imatinib (STI571)	- Inhibits ligand induced PDGFR- α phosphorylation -	-Inhibits growth of nerve sheath tumors.	- N.S.	- N.S.	- (Holtkamp et al., 2005)

	Means of inhibition	Effect on PDGFR-CC pathway	Other biological functions tested	Effect on BBB	Effect on neurodegeneration	Reference
				- Reduced stroke induced BBB opening	- Reduced stroke infarct size edema and hemorrhage.	- (Su et al., 2008) (Ma et al., 2011) (Zhan et al., 2015)
				- N.S.	Reduced kainate induced seizure onset and progression.	- (Fredriksson et al., 2015)
				-Reduced spinal cord trauma induced BBB opening	- Reduced glial scar, improved locomotor performance	- (Abrams et al., 2012) (Sharp et al., 2014)
				-Reduced TBI induced BBB opening	- Reduced edema, lesion volume, tissue and memory loss	- (Su et al., 2015)
				-Reduced ALS associated BBB dysfunction	- Reduced motor neuron loss and delayed ALS onset	- (Lewandowski et al., 2016)
				- Reduced EAE associated BBB dysfunction	- Reduced CNS inflammation and demyelination	- (Adzemovic et al., 2013)
	Sunitinib (SU11248)	- Inhibits PDGFR- α auto-phosphorylation	- Inhibits growth of transformed Ba/F3 cells	- N.S.	- N.S.	- (Prenen, 2006)
				- N.S.	-Reduces hypoxia induced BBB opening and edema	- (Saraswat et al., 2015)
	Nilotinib (AMN107)	- Inhibits PDGFR- α auto-phosphorylation	- Inhibits growth of transformed Ba/F3 cells	- N.S.	- N.S.	- (Stover et al., 2005)
				- N.S.	- Reduces neuronal loss, motor and cognitive decline in ALS mice	- (Wenqiang et al., 2014)
				- N.S.	- Neuroprotective and improves motor performance in α -synuclein PD model	- (Hebron et al., 2013)
				- N.S.	-Prevents neuronal loss and behavioral deficits in MPTP induced PD model	- (Karuppagounder et al., 2014)
	Dasatinib (BMS-354825)	- Inhibits PDGFR- α auto-phosphorylation	- Inhibits growth of PDGFR- α DIM842-844 transformed Ba/F3 cells	- N.S.	- N.S.	- (Dewaele et al., 2008)
				- N.S.	- Improves memory, decreased microglia activation in AD mice.	- (Dhawan & Combs, 2012)
				- N.S.	- Delays onset of EAE	- (Azizi et al., 2015)
				- N.S.	- Extends survival and motor skills in ALS mice	- (Katsumata et al., 2012)

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	Means of inhibition	Effect on PDGF-CC pathway	Other biological functions tested	Effect on BBB	Effect on neurodegeneration	Reference
	Masitinib (AB1010)	- Inhibits PDGFR- α auto-phosphorylation	- Inhibits growth of FIP1L1 - PDGFR- α transformed Ba/F3 cells	- N.S.	- N.S.	- (Sadovnik et al., 2014)
				- N.S.	- Neuroprotective in ischemic stroke	- (Kocic et al., 2015)
				- N.S.	- Beneficial in AD trial	- (Piette et al., 2011)
				- N.S.	- Beneficial in MS trial	- (Vermersch et al., 2012)
				- N.S.	- Report of use in an ALS trial	- (Salvado et al., 2015)