Altered Blood-Brain Barrier and Blood-Spinal Cord Barrier Dynamics in Amyotrophic Lateral Sclerosis: Impact on Medication Efficacy and Safety

Yijun Pan[1] and Joseph Nicolazzo[1]

[1]Monash Institute of Pharmaceutical Sciences, Monash University

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Abstract

The access of drugs into the central nervous system (CNS) is regulated by the blood-brain barrier (BBB) and blood-spinal cord barrier (BSCB). A large body of evidence supports perturbation of these barriers in neurodegenerative diseases, including Alzheimer's disease and Parkinson's disease. Modifications to the BBB and BSCB are also reported in amyotrophic lateral sclerosis (ALS), albeit these modifications have received less attention relative to those in other neurodegenerative diseases. Such alterations to the BBB and BSCB have the potential to impact on CNS exposure of drugs in ALS, modulating the effectiveness of drugs intended to reach the brain and the toxicity of drugs that are not intended to reach the brain. Given the clinical importance of these phenomena, this review will summarise reported modifications to the BBB and BSCB in ALS, discuss their impact on CNS drug exposure and suggest further research directions so as to optimise medicine use in people with ALS.

Altered Blood-Brain Barrier and Blood-Spinal Cord Barrier Dynamics in Amyotrophic Lateral Sclerosis: Impact on Medication Efficacy and Safety

Yijun Pan, Joseph A Nicolazzo*

Drug Delivery, Disposition and Dynamics, Monash Institute of Pharmaceutical Sciences, Monash University, 399 Royal Parade, Parkville, Victoria 3052, Australia.

* Corresponding author

Ph: +61 3 9903 9605, Fax: +61 3 9903 9583

E-mail: joseph.nicolazzo@monash.edu

LIST OF ABBREVIATIONS

ABC proteins	ATP-binding cassette (ABC) proteins
AD	Alzheimer's disease
ALS	amyotrophic lateral sclerosis
BBB	blood-brain barrier
BCRP	breast cancer resistance protein
BSCB	blood-spinal cord barrier
CNS	central nervous system
GLUT1	glucose transporter 1
JAM	junctional adhesion molecule
LAT1	L-type amino acid transporter 1

ABC proteins	ATP-binding cassette (ABC) proteins
PD	Parkinson's disease
P-gp	P-glycoprotein
SOD1	superoxide dismutase 1
TDP43	TAR DNA-binding protein 43
TJ	tight junction
ZO	zonula occludens

ABSTRACT

The access of drugs into the central nervous system (CNS) is regulated by the blood-brain barrier (BBB) and blood-spinal cord barrier (BSCB). A large body of evidence supports perturbation of these barriers in neurodegenerative diseases, including Alzheimer's disease and Parkinson's disease. Modifications to the BBB and BSCB are also reported in amyotrophic lateral sclerosis (ALS), albeit these modifications have received less attention relative to those in other neurodegenerative diseases. Such alterations to the BBB and BSCB have the potential to impact on CNS exposure of drugs in ALS, modulating the effectiveness of drugs intended to reach the brain and the toxicity of drugs that are not intended to reach the brain. Given the clinical importance of these phenomena, this review will summarise reported modifications to the BBB and BSCB in ALS, discuss their impact on CNS drug exposure and suggest further research directions so as to optimise medicine use in people with ALS.

Introduction

Amyotrophic lateral sclerosis (ALS) is the most common motor neurone disease in adults, leading to muscle weakness and eventual paralysis and respiratory failure. ALS typically occurs in late middle life (51-66 years), with an incidence of 0.6-3.8 per 100 000 person-years and a prevalence of 4.1-8.4 per 100 000 persons (Longinetti & Fang, 2019). Despite affecting individuals for more than 150 years, this progressive neurodegenerative and fatal disease has limited treatment options, with riluzole and edaravone being the only two United States Food and Drug Administration-approved treatment options (Shefner et al., 2020). The majority of individuals with ALS can survive 3-5 years after appearance of symptoms, despite being administered riluzole (Chiò et al., 2009). ALS is mainly a sporadic disease with about 10% of cases being familial in nature (Chiò et al., 2009). Multiple factors contribute to the pathogenesis of ALS, including dysfunctional RNA metabolism, defective protein homeostasis, mitochondrial dysfunction, oxidative stress, neuroinflammation, and vesicular transport defects (Mejzini, Flynn, Pitout, Fletcher, Wilton & Akkari, 2019).

Despite a wealth of research being undertaken to reverse the pathology associated with ALS, translating this to ALS therapeutics has not been very successful. While there are multiple reasons for preclinical-to-clinical failure for ALS therapeutics, including heterogeneity of ALS pathology (Beghi et al., 2007) and species differences in efficacy, a major reason as to why many CNS candidates fail to reach the market is their inability to reach their site of action within the brain, due to the defence nature of the CNS barriers (Nicolazzo, Charman & Charman, 2006). These barriers, including the blood-brain barrier (BBB) and blood-spinal cord barrier (BSCB), consist of a layer of endothelial cells connected by tight junction (TJ) proteins to limit paracellular transport and express influx and efflux transporters that precisely control permeation of circulating solutes including drugs (Abbott, Patabendige, Dolman, Yusof & Begley, 2010). In neurodegenerative disorders such as Alzheimer's disease (AD) and Parkinson's disease (PD), there are multiple reports of altered BBB ultrastructure and permeability, which have been reported to affect CNS drug delivery (Pan & Nicolazzo, 2018).

Modifications to the BBB and BSCB are also reported in ALS and such modifications have the potential to impact on CNS exposure of drugs, including those that are intended to reach the brain and those for which CNS exposure leads to off-target effects. As well as riluzole and/or edaravone, individuals with ALS are prescribed 5-10 medicines intended to reach the brain for management of common ALS symptoms such as

cramps, spasticity and pain (Meyer et al., 2020). In the last 12 months of life, 57.4% and 44.1% of individuals with ALS are prescribed psychoanaleptics and psycholeptics, respectively (Grande, Morin, Vetrano, Fastbom & Johnell, 2017), and any BBB/BSCB changes could impact on their CNS exposure and efficacy. In addition, individuals with ALS are often afflicted with various comorbidities including hypertension, diabetes and hypercholesterolaemia requiring medicines that do not require access into the brain (Hobson & McDermott, 2016). Any alterations to the BBB/BSCB in ALS could result in undesirable CNS accumulation of these drugs, leading to unintended neurotoxicity in people with ALS.

Therefore, characterising the status of the CNS barriers in ALS is important for both drug discovery and for optimising medicine use in individuals with ALS. A better characterisation of the status of the BBB/BSCB can guide the design of CNS barrier-targeting approaches to enhance CNS access of novel preclinical candidates for ALS. On the other hand, appreciating the status of the CNS barriers in ALS could assist in implementing strategies to minimise the CNS exposure of drugs not intending to reach the brain, so as to minimise undesirable adverse effects and ultimately improving quality of life. This review will provide a general introduction to the structure and function of CNS barriers, highlight modifications of CNS barriers in ALS and discuss how these modifications have the potential to impact on drug development and medication safety and effectiveness in ALS.

The CNS barriers

2.1 BBB and BSCB physiology

The CNS barriers are formed by a layer of endothelial cells separating the blood and the CNS parenchyma (**Figure 1**), with the BBB and BSCB protecting the brain and spinal cord, respectively, from blood-derived toxins. The endothelial cells are connected by inter-endothelial TJ and adhesion proteins. A basement membrane supports the brain or spinal cord endothelial cell and associated pericytes, with astrocytic end-feet ensheathing the basement membrane (Abbott, Patabendige, Dolman, Yusof & Begley, 2010) (**Figure 1**).

Figure 1. A schematic diagram illustrating the constituents of the CNS barriers. The endothelial cells are connected by tig

The defence characteristics of the CNS barriers can be considered as both physical and biochemical barriers. The physical barrier is imparted by TJs, which are formed through a sophisticated network of interacting proteins, such as occludin, claudins, junctional adhesion molecules (JAMs) and zonula occludens, which prevent the paracellular movement of solutes (Abbott, Patabendige, Dolman, Yusof & Begley, 2010). The biochemical barrier is imparted by the function of efflux transporters, such as the ATP-binding cassette (ABC) proteins, P-glycoprotein (P-gp) and breast cancer resistance protein (BCRP), that effectively exclude various endogenous and exogenous toxins from the endothelial cells. In addition to the physical and biochemical barriers, there exist a number of influx transporters, such as glucose transporter 1 (GLUT1) and large amino acid transporter (LAT1), which facilitate the uptake of essential nutrients into the CNS (Abbott, Patabendige, Dolman, Yusof & Begley, 2010). The BSCB is slightly more permeable than the BBB in mice (Pan, Banks & Kastin, 1997), likely attributed to reduced abundance of zonula occludens-1 and occludin (Ge & Pachter, 2006). Furthermore, the levels of efflux transporters and TJ proteins were reported higher in the human BBB relative to the BSCB (Uchida et al., 2020).

2.2 CNS barriers and drug delivery

The presence of efflux drug transporters such as P-gp and BCRP at CNS barriers, in addition to the TJ architecture, are known to limit the CNS access of drugs, reducing their ability to reach their site of action within the brain (Nicolazzo, Charman & Charman, 2006). Pharmacological inhibition of P-gp has been trialled as an approach to improve CNS drug delivery; however, this approach has not been clinically-translated due to the undesirable effects associated with blanket suppression of P-gp function (Davis, Sanchez-Covarubias & Tome, 2014). On the other hand, influx transporters have been targeted to improve CNS drug delivery. For example, drugs and prodrugs (e.g. L-DOPA, melphalan, and gabapentin)

have been designed to be recognised as substrates to LAT1 (Puris, Gynther, Auriola & Huttunen, 2020), leading to improved brain uptake and effectiveness.

While CNS barriers are considered a major barrier to drug delivery under healthy conditions, it is becoming increasingly apparent that these barriers are modulated in various diseases of the CNS, such as AD and PD (Pan & Nicolazzo, 2018). This has been shown to lead to altered drug access to the CNS, potentially resulting in a loss of desirable pharmacological effects or an increased risk of CNS adverse effects. While there has been significant focus on CNS barrier modifications in AD and PD (Al-Bachari, Naish, Parker, Emsley & Parkes, 2020; Sweeney, Sagare & Zlokovic, 2018), less studies have focused on modification to the CNS barriers in ALS. Therefore, this review will (i) provide a contemporary update on the status of the BBB and BSCB in ALS, (ii) provide insight into the potential impact of these changes on CNS drug exposure and medicine use in people with ALS, and (iii) suggest recommendations for future research directions to ensure effective CNS drug delivery and medicine use in ALS.

Modification of CNS barriers in ALS

Many of the studies reporting altered CNS barrier function in sporadic ALS are based on observations using post mortem human tissues, while transgenic rodent models expressing mutant genes, e.g. superoxide dismutase 1 G93A ($SOD1^{G93A}$) have significantly contributed to our understanding of CNS barrier changes in familial ALS (summarised in **Table 1** , **Table 2** and graphically presented in **Figure 2**).

3.1 Evidence from post-mortem human tissues

3.1.1 Ultrastructural modifications

In postmortem ALS human spinal cord, electron microscopy and immunohistochemistry revealed swelling and cytoplasmic vacuolisation of microvascular endothelial cells, degeneration of pericytes and detachment of astrocyte end-feet processes from endothelial cells (Garbuzova-Davis et al., 2012; Miyazaki et al., 2011; Sasaki, 2015; Yamadera et al., 2015). Studies also reported increased (Garbuzova-Davis et al., 2012; Waters et al., 2021) or decreased (Miyazaki et al., 2011; Ono et al., 1998) collagen content in the basement membrane of the spinal cord microvasculature in ALS patients. Reduced collagen content in the basement membrane can be to due cellular damage, whereas a thickened basement membrane could be a result of repetitive regeneration. This discrepancy in the above reports could be attributed to the different CNS regions assessed, the heterogeneity in ALS pathology, and varied quantification methods employed to assess collagen content. Further studies are required to confirm this in a more systematic manner, and assess the impact of basement thickening or thinning on CNS exposure of passively-diffusing compounds, given that brain microvascular basement membrane thickening has been associated with reduced BBB transport of passively-diffusing drugs in a mouse model of AD (Mehta, Short & Nicolazzo, 2013).

3.1.2 Reduced expression of TJ and adhesion proteins

Decreased expression of TJ and adhesion proteins in spinal cords from ALS patients has been confirmed using PCR and Western Blot. Henkel*et al.* assessed mRNA expression of zonula occludens-1, occludin, and claudin-5 in lumbar spinal cord homogenates from individuals with ALS (30 sporadic and 4 familial cases) and 16 non-ALS controls (Henkel, Beers, Wen, Bowser & Appel, 2009). A significant reduction in zonula occludens-1 and occludin levels were identified in sporadic ALS and familial ALS, respectively, while claudin-5 was unchanged in both sporadic and familial ALS. In another study, a significant decrease in most TJ and adhesions proteins were reported in ALS spinal cord (cervical and lumbar) relative to non-ALS controls, including zonula occludens-1, occludin, claudin-5, JAM-1 and VE-cadherin (Garbuzova-Davis et al., 2012). Despite decreased TJ protein expression at the ALS BSCB, the junction morphology was generally well-preserved as assessed by electron microscopy (Sasaki, 2015). Based on these TJ and adhesion junction reductions, it would be predicted that spinal cord access of molecules would be increased as a result of increased paracellular permeability.

3.1.3 Leaky CNS barriers

For human post mortem studies, the leakiness of CNS barriers is often inferred from the presence of plasma-derived proteins in brain or spinal cord. Donnenfeld *et al.* identified IgG and C3/C4 complement in the motor cortex and spinal cord of ALS patients nearly 40 years ago (Donnenfeld, Kascsak & Bartfeld, 1984). These deposits were detected immunohistochemically in 5 of 13 ALS motor cortices and 6 of 16 ALS spinal cords, but not in non-ALS controls. Findings by Donnenfeld*et al.* were later confirmed by Engelhardt *et al* . immunohistochemically, where IgG was identified in 13 of 15 ALS spinal cords and 6 of 11 ALS motor cortices (Engelhardt & Appel, 1990). In addition to IgG, Winker *et al.* noted haemoglobin, fibrinogen and thrombin in the ALS cervical spinal cord (8 sporadic ALS cases, 3 familial ALS cases) but not in non-ALS controls (5 cases) (Winkler, Sengillo, Sullivan, Henkel, Appel & Zlokovic, 2013). In line with these postmortem findings, increased IgG (1.26-fold) and albumin (1.28-fold) has been reported by Leonardi *et al.* (Leonardi, Abbruzzese, Arata, Cocito & Vische, 1984) in ALS cerebrospinal fluid (CSF) (90 ALS cases, 50 controls) and elevated haemoglobin levels (1.86-fold) has been reported in the CSF of individuals with ALS by Waters *et al.* (236 ALS cases, 87 controls) (Waters *et al., 2021)* . Consistently, these studies demonstrate that CNS barrier permeability is generally increased in individuals with ALS, which is very likely a result of the ultrastructural abnormality and reduced expression of TJs at the ALS CNS barriers. While these modifications likely contribute to the leakiness of endogenous proteins into the CNS, the impact of these changes on the CNS access of small and large molecular weight therapeutics has yet to be undertaken clinically. This could be achieved using imaging techniques such as magnetic resonance imaging to visualise CNS access of gadolinium in ALS.

3.1.3 Increased expression of P-gp and BCRP

The cellular distribution and expression of P-gp and BCRP has been assessed in the cervical spinal cord and motor cortex obtained from individuals with ALS. Using immunohistochemistry, van Vliet *et al.* has demonstrated a dramatic (˜30-fold) increase in P-gp abundance in cervical spinal cord and motor cortex astrocytes in ALS tissues relative to control specimens, while P-gp abundance at the endothelial cell lining of microvessels was comparable between control and ALS spinal cord and motor cortex specimens (van Vliet et al., 2019). A similar study was conducted by Jablonski *et al.* , who reported an elevated P-gp abundance in microvessels from ALS lumbar spinal cord (Jablonski et al., 2012). The discrepancies between these studies could be due to different CNS regions assessed (cervical spinal cord, lumbar spinal cord, and motor cortex). In terms of BCRP, microvasculature expression was observed in both control and ALS specimens, and its abundance did not differ between control and ALS in the cervical spinal cord but was marginally increased in the ALS motor cortex (van Vliet et al., 2019).

Further studies are required to confirm these findings and possibly improve our understanding of CNS barriers protein expression in ALS, i.e. beyond P-gp and BCRP. Specialised microvascular isolation techniques can be implemented to obtain high purity of microvessels or isolated endothelial cells, which can be analysed via proteomic approaches. Such approaches may identify modified expression of efflux and influx transporters in ALS, which can assist in (i) targeting transporters to increase CNS exposure of otherwise impermeable compounds and (ii) guiding dosage regimen design of medicines which are substrates for such transporters to avoid potential CNS toxicity. Furthermore, the functional consequence of these expression changes on CNS drug exposure in humans is completely lacking, and could be confirmed using positron emission tomography studies with ^{11}C-verapamil (as a substrate of P-gp) as has been undertaken in humans with AD and PD (Bartels et al., 2008; Lubberink, van Assema, Hendrikse, Schuit, Lammertsma & Van Berckel, 2010).

Table 1. Evidence of CNS barrier modifications in ALS from human biospecimens.

Parameter	**Observation**	**References**
Ultrastructure	morphological changes in endothelium, astrocytes, pericytes	Miyazaki 2011, Garbuzova-Davis 2012, Winkler 2013, Sasaki 2015, Yamadera 2015
	| collagen content	Garbuzova-Davis 2012, Sasaki 2015, Waters 2021

	| collagen content	Ono 1998, Miyazaki 2011
Tight junctions	well-preserved structure (electron microscopy)	Sasaki 2015
	| mRNA or protein expression	Henkel 2009, Garbuzova-Davis 2012
Leakiness	blood derived protein detected in the spinal cord/cortex/CSF	Donnefeld 1984, Leonardi 1984, Engelhardt 1990, Garbuzova-Davis 2012, Winkler 2013, Waters 2012
Efflux transporters P-gp	| (spinal cord homogenates) no change (spinal cord and motor cortex endothelium) | (spinal cord and motor cortex astrocytes)	Jablonski 2012 van Vliet 2019
BCRP	| in spinal cord homogenates | (motor cortex endothelium); | (spinal cord and motor cortex astrocytes)	Jablonski 2012 van Vliet 2019

3.2 Evidence from rodent models

3.2.1 Transgenic ALS rodent models used for evaluation of CNS barriers

In order to provide a review of the changes to CNS barriers in animal models of ALS, it is important to describe the commonly-used models of ALS. The human $SOD1^{G93A}$ transgenic mouse model, which harbors a mutation in human SOD1, was the first established animal model for ALS (Gurney et al., 1994; Rosen et al., 1993), which recapitulates many human ALS symptoms; mice first demonstrate a hindlimb tremor, followed by loss of the hindlimb splaying reflex, and eventually paralysis and death. Numerous SOD1 transgenic models of ALS were designed thereafter using patient sequence variants from genomic fragments, including the G37R mutation (Wong et al., 1995), the G86R mutation (Bartels et al., 2008), the G85R mutation (Bruijn et al., 1997), the L126Z mutation (Wang et al., 2005), and the T116X mutation (Han-Xiang et al., 2008). While each line differs in onset, presentation, and progression, SOD1 overexpressing transgenic mice generally share traits of significant motor neuron loss, axonal denervation, progressive paralysis, and reduced lifespan (Philips & Rothstein, 2015; Picher-Martel, Valdmanis, Gould, Julien & Dupré, 2016). In addition to the mouse model, a transgenic $SOD1^{G93A}$ rat model of ALS has been developed and validated, exhibiting a more rapid progression of disease (Howland et al., 2002). However, SOD1 models are based off a small fraction of an extremely heterogeneous disease, as only ~2% of human ALS (familial and sporadic) is due to SOD1 mutations (Picher-Martel, Valdmanis, Gould, Julien & Dupré, 2016). Therefore, findings obtained with this model need to be carefully considered as they may not be replicated in the majority of individuals with ALS.

While TAR DNA-binding protein 43 (TDP43) mutations only account for less than 5% of familial ALS cases, its identification as a causative gene to ALS in 2008 resulted in ~ 20 mouse models (Picher-Martel, Valdmanis, Gould, Julien & Dupré, 2016). The $TDP43^{A315T}$ mouse was the first reported TDP43 mutation-based ALS mouse model (Wegorzewska, Bell, Cairns, Miller & Baloh, 2009), which develops a gait abnormality, significant weight loss and a characteristic "swimming" gait.

While there exist other mouse models of ALS, such as $FUS^{1\text{-}359}$ mice (Shelkovnikova et al., 2013) and C9orf72-based mouse models (Jiang et al., 2016; O'Rourke et al., 2015; Peters et al., 2015), they will not be described in detail as no studies assessing BBB or BSCB function have been reported in these animal models.

3.2.2 Ultrastructural abnormality

Similar to individuals with ALS, altered ultrastructure of CNS barriers has been reported in SOD1^{G93A} mice (Garbuzova-Davis, Haller, Saporta, Kolomey, Nicosia & Sanberg, 2007). Brain stem and cervical and lumbar spinal cords of SOD1^{G93A} mice were assessed using electron microscopy, and degenerating endothelial cells, vacuolated endothelial cytoplasm, and swollen astrocyte end-feet were noted at an early disease stage that worsened with disease progression. A thickened basement membrane was noted around the severely degenerated endothelia of all three regions assessed, suggesting a reparative process (Garbuzova-Davis, Haller, Saporta, Kolomey, Nicosia & Sanberg, 2007). In their subsequent study, reduced laminin-1 (major non-collagenous basement membrane glycoprotein) was demonstrated immunohistochemically in the BSCB of SOD1^{G93A} mice at both early and late stages (Garbuzova-Davis et al., 2007). In line with this, reduced and undetectable collagen IV content was reported in the BSCB basement membrane of SOD1^{G93A} mice (Miyazaki et al., 2011). These ALS-related modifications at the BSCB were also identified in SOD1^{G93A} rats (Nicaise et al., 2009). Despite progression of ALS pathology being more rapid in the SOD1^{G93A} rat model, ultrastructural alterations were only observed in symptomatic SOD1^{G93A} rats but not pre-symptomatic rats. Overall, these studies suggest a compromised BSCB structural integrity in SOD1^{G93A} models, and that this may lead to increased penetration of drugs into the spinal cord, however, functional studies to confirm this hypothesis are lacking. Furthermore, little information is available on any potential ultrastructural changes at the BBB of the motor cortex in SOD1^{G93A} mice, an area which is important to investigate to appreciate the impact of the disease on brain access of drugs.

3.2.3 Reduced expression of TJ proteins

Using electron microscopic analysis, Garbuzova-Davis *et al.*suggested that the endothelial cell TJs appeared intact in early and late symptomatic SOD1^{G93A} mice (Garbuzova-Davis, Haller, Saporta, Kolomey, Nicosia & Sanberg, 2007). However, using a more quantitative approach (Western blot), a 30-60% reduction in zonula occludens-1, occludin and claudin-5 was consistently observed in spinal cord microvessels of SOD1^{G93A} mice relative to wildtype mice (Zhong et al., 2008). In addition, a progressive reduction in occludin abundance at the BSCB in SOD1^{G93A} mice was demonstrated by Miyazaki *et al.* using immunohistochemistry and Western blot (Miyazaki et al., 2011). Similarly, in SOD1^{G93A} rats, zonula occludens-1 and occludin mRNA were significantly reduced in the lumbar spinal cords at the symptomatic stage only but not in the brain or brain stem (Nicaise et al., 2009).

3.2.4 Leaky CNS barriers

An increased paracellular permeability of the BSCB was clearly demonstrated by intravenous injection of Evans Blue, a tracer used to assess barrier disruption, in SOD1^{G93A} mice (Garbuzova-Davis et al., 2007). Vascular leakage (indicated by Evans Blue extravasation) was observed in lumbar spinal cord, and cervical spinal cord from 13 weeks of age in SOD1^{G93A} mice. Considerable vessel permeability was observed in early as well as late stage symptomatic SOD1^{G93A} mice. Notably, more vascular leakage was detected in the lumbar spinal cord than cervical spinal cord at the late stage of disease. Using Evans Blue again, vessel leakiness has been demonstrated in the spinal cord and brain stem, but not the brain, of SOD1^{G93A} rats at symptomatic stages (Nicaise et al., 2009). However, using a sensitive magnetic resonance imaging approach, increased BBB permeability was demonstrated in the brains of SOD1^{G93A} rats (Andjus et al., 2009). Overall, these studies demonstrate increased permeability of CNS barriers in rodent models of ALS, which is expected based on reduced TJ expression in these models. However, whether such changes lead to altered drug penetration into the spinal cord or brain of ALS mice remains to be investigated. It would be hypothesised that drug penetration would increase in mouse models of ALS based on the reported TJ dysfunction and paracellular, however, the modifications in efflux transporter expression (described below) may counteract this leakiness, and indeed reduce CNS exposure of drugs.

3.2.4 Increased expression and activity of P-gp and BCRP

A selective increase in microvascular expression of P-gp and BCRP during disease progression in three ALS mouse models (SOD1^{G93A}, SOD1^{G86R} and TDP43^{A315T}) has been reported in the cerebral cortex and spinal cord (Jablonski et al., 2012; Milane et al., 2010). mRNA levels were also significantly increased in lumbar

spinal cord homogenates 2.13-fold for P-gp and 1.72-fold for BCRP in mice at symptomatic stages compared to those at presymptomatic stages (Jablonski et al., 2012). In addition, Western blot was employed to assess P-gp and BCRP abundance in the whole spinal cord and cerebral cortex of $SOD1^{G93A}$ mice. Increased expression of P-gp (1.96-fold) and BCRP (1.69-fold) was observed in the spinal cord of symptomatic mice compared to presymptomatic $SOD1^{G93A}$mice and elevated levels of P-gp (1.35-fold) and BCRP (1.28-fold) were reported in the cerebral cortex of symptomatic mice compared to presymptomatic $SOD1^{G93A}$ mice (Jablonski et al., 2012). The increased expression of P-gp and BCRP was demonstrated in another mouse model of ALS by Jablonski *et al.* comparing P-gp and BCRP levels in the spinal cord and cerebral cortex of symptomatic $TDP43^{A315T}$ mice with presymptomatic mice. An independent group (Milane et al., 2010) demonstrated increased P-gp abundance (1.5-fold) in microvessels isolated from the brains of presymptomatic $SOD1^{G86R}$ mice compared to age-matched wildtype controls. However, no alteration of BCRP abundance was detected in these $SOD1^{G86R}$ mice.

In $SOD1^{G93A}$ rats, microvessels from brain and spinal cord sections were assessed immunohistochemically and microvessels were also isolated for protein quantification using Western blot (Chan, Evans, Banks, Mesev, Miller & Cannon, 2017). In this study Chan*et al* . demonstrated immunohistochemically that P-gp protein expression was significantly increased in the cerebral cortex (1.88-fold) and the spinal cord (1.46-fold) from symptomatic $SOD1^{G93A}$ rats compared to age-matched wildtype rats. In addition, the expression of BCRP was not affected when assessed immunohistochemically. Given immunohistochemistry is semi-quantitative, protein expression in isolated microvessels was reassessed using Western blot, and a 1.5-fold increase in P-gp expression was demonstrated in isolated microvessels from brain and spinal cord of symptomatic $SOD1^{G93A}$ rats. It was also noted that microvascular BCRP expression increased 1.15- and 1.25-fold in brain and spinal cord, respectively, although statistical significance was not achieved. These studies clearly demonstrate increased expression of P-gp and sometimes BCRP in the brain and spinal cord in models of ALS. Some differences in the results were observed. For example, whole brain and spinal cord homogenates were used by Jablonski *et al* . and the samples obtained from the microvascular isolation techniques employed by Chan*et al.* and Milane *et al.* often contain astrocytes, which express P-gp (Golden & Pardridge, 1999).

In addition to assessing expression of P-gp and BCRP, Jablonski *et al.* assessed their activity using a confocal microscopy-based transport assay where microvessles were incubated with fluorescent substrates (NBD-cyclosporin A for P-gp; bodipy-prazosin for BCRP) (Jablonski et al., 2012). Luminal substrate accumulation was measured in the absence and presence of specific transport inhibitors (PSC833 for P-gp, Ko143 for BCRP). This assay successfully demonstrated that the transport activity of P-gp was increased 1.8-fold and 2-fold in brain and spinal cord microvessels, respectively, from symptomatic $SOD1^{G93A}$ mice relative to age-matched wildtype mice and presymptomatic $SOD1^{G93A}$ mice. In a follow up study, the *in vivo* accumulation of a specific P-gp substrate, LD800, in the spinal cord of symptomatic $SOD1^{G93A}$ mice was assessed and compared to wildtype mice following intraperitoneal administration (Jablonski et al., 2014). Spinal cord levels of LD800 levels were significantly lower in $SOD1^{G93A}$ mice comparing to wildtype mice, suggesting increased P-gp activity at the BSCB, in line with increased P-gp expression. A similar study was performed, where a P-gp substrate [^{3}H]-digoxin and a BCRP substrate [^{3}H]-prazosin were dosed intraperitoneally to both $SOD1^{G86R}$ mice and wildtype mice (Milane et al., 2010). A reduced disposition of [^{3}H]-digoxin (1.5-fold) but not [^{3}H]-prazosin was observed in the brain of presymptomatic $SOD1^{G86R}$ mice compared to wildtype controls, suggesting increased function of P-gp but not BCRP in this mouse model. This alteration is in line with reduced P-gp protein abundance (1.5-fold) and unchanged BCRP abundance observed in this mouse model.

Chan *et al.* demonstrated a 2-fold increase in P-gp activity in microvessels isolated from the brain and spinal cord of symptomatic $SOD1^{G93A}$ rats compared to those from presymptomatic rats using the same confocal microscopy-based transport assay mentioned above (Chan, Evans, Banks, Mesev, Miller & Cannon, 2017). Overall, these studies demonstrate increased P-gp and BCRP abundance and activities at the CNS barriers in ALS rodent models, suggesting that the barriers may be more restrictive to substrates of these transporters. This is of particular concern given that riluzole, which is effective in early ALS and less effective in late stage ALS, is a substrate of P-gp and BCRP (Zoccolella et al., 2007). The reduced effectiveness of riluzole

in SOD1^{G93A} rats may result from P-gp/BCRP overactivity, as less riluzole may be able to enter the CNS. In fact, a 1.7-fold reduction in riluzole disposition into the brain of SOD1^{G86R} has been reported compared to wildtype mice following intraperitoneal administration (Milane et al., 2010). Therefore, approaches to overcome increased P-gp and BCRP activities in ALS may improve the effectiveness of riluzole. This has been confirmed in SOD1^{G93A} mice that received riluzole with or without elacridar (an inhibitor of P-gp and BCRP) (Jablonski et al., 2014). The efficacy of riluzole improved when co-administered with elacridar and this was associated with increased riluzole delivery to the brain. However, such generic inhibition of P-gp/BCRP comes with side effects, and therefore, an approach to restore P-gp/BCRP to levels similar to the healthy CNS barriers is more likely to be a more effective clinical strategy. In addition, while much has been reported on P-gp and BCRP in ALS, our knowledge on other transporters at the CNS barriers is limited. Profiling the expression at the CNS barriers could potentially identify efflux transporters that should be avoided for effective CNS delivery or influx transporters, which could be effectively targeted to increase CNS exposure of drugs intended to reach the CNS.

Table 2. Evidence of CNS barrier modifications in ALS from rodent models.

Parameter	Observation	References
Ultrastructure	morphological changes to endothelium, astrocytes, and pericytes	Garbuzova-Davis 2007a, Nicaise 2009
	| structural proteins in the basement membrane (e.g. collagen IV, laminin-1)	Garbuzova-Davis 2007b, Miyazaki 2011, Nicaise 2009
Tight junctions	| mRNA or protein expression	Miyazaki 2011, Zhong 2008, Nicaise 2009
Leakiness	| Evans Blue extravasation	Garbuzova-Davis 2007b, Nicaise 2009
Efflux transporter P-gp	| expression and activity	Milane 2010, Jablonski 2012, Jablonski 2014, Chan 2017
BCRP	no change to expression and activity	Milane 2010, Chan 2017
	| expression and activity	Jablonski 2012

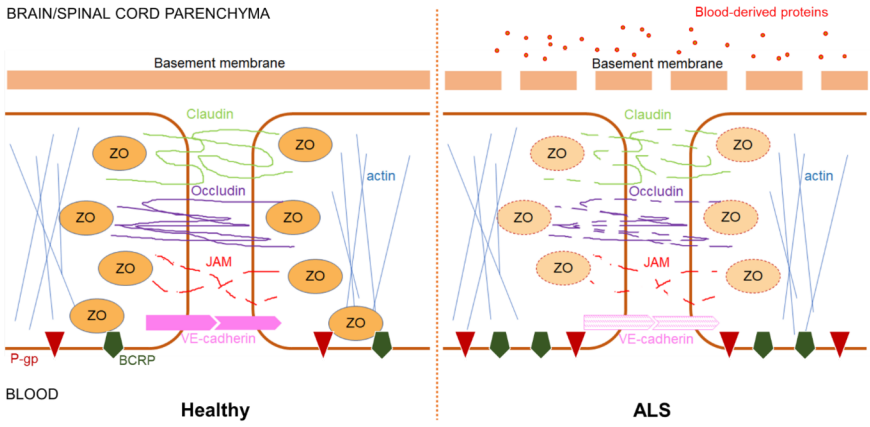

Figure 2 . A summary of the modifications to TJ, adhesion junctions and efflux transporter expression at ALS CNS barriers. The abundance of TJ and adhesion proteins are generally reduced, leading to a compromised junctional integrity and increase in paracellular permeability. The basement membrane thickness

is reported to either increase as a reparative mechanism or decrease with reduced collagen and laminin in ALS, with discrepancies reported in human tissues, while being consistently decreased in animal models. The expression of P-gp and BCRP are generally increased in ALS, which is expected to result in reduced CNS exposure of drugs that are substrates for these efflux transporters. ZO: zonula occludens, P-gp: P-glycoprotein, BCRP: breast cancer resistance protein, JAM: junctional adhesion molecules.

Summary and future directions

Human and rodent studies have consistently demonstrated ultrastructural abnormality, increased paracellular permeability and elevated P-gp (and sometimes BCRP) expression and activity at the CNS barriers in ALS. Based on the reports of reduced TJ function, it would be expected that general CNS permeability to drugs would be elevated in ALS, however, this may be counteracted especially if the drugs are substrates of P-gp and/or BCRP. Furthermore, if there is indeed a thickening of the cerebral or spinal microvasculature basement membrane, this could result in lower brain and spinal cord access of drugs as has been reported in a mouse model of AD with thickened cerebrovascular basement membrane (Mehta, Short & Nicolazzo, 2013). Therefore, it is clear that a more detailed functional analysis of transport processes of many drugs which are trafficked via different mechanisms (i.e. paracellular, transcellular, substrates of influx and efflux transporters) is required so as to predict how CNS access of drugs indeed alters in ALS.

With disease progression, the expression and activity of P-gp and BCRP generally increases, which may lead to suboptimal drug delivery, while this yet to be confirmed in people with ALS, for example, by using PET imaging. If this is validated in humans, specialised approaches can be trialled to improve the CNS access of riluzole (and other CNS-acting drugs) to improve therapeutic outcomes. This can be via pharmacological manipulation of P-gp expression and activity or by transiently disrupting the CNS barriers using emerging technology such as MR-guided focused ultrasound (Abrahao et al., 2019).

Our current understanding of the CNS barriers in ALS is still limited. The majority of studies have only investigated P-gp and BCRP expression. Proteomic studies can be performed using microvessels isolated from post-mortem ALS human brain/spinal cords or from transgenic ALS mice, to generate a more detailed status of the ALS BBB and BSCB. A better appreciation of the status of BBB/BSCB influx transporters in ALS can assist in the design of new chemical entities that can specifically target these influx transporters to enhance CNS exposure of otherwise impermeable drugs. These studies will also highlight, based on their affinity to transporters, which drugs not intended to reach the CNS have increased CNS access in ALS, informing which drugs may require dosage adjustment so as to avoid excessive CNS exposure. Ultimately, this will guide optimum dosing in individuals for all medications consumed by people with ALS to maximise effectiveness (when CNS access is required) and minimise CNS toxicity (when CNS access is not desirable), overall enhancing optimum use of medicines in individuals with ALS.

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