1.8.1 Protein tyrosine phosphatase family

Protein tyrosine phosphatases (PTPs) are involved in a multitude of signalling cascades to regulate cellular processes including growth, differentiation, mitosis and transformation. The first member of the PTP family (PTP1B) was discovered in the human placenta [345]. The PTP family consists of four classes, Class I-III and the Class IV, which is also known as aspartate based. All PTPs, excluding class IV, contain a conserved active C(X)5R motif site and share a similar catatlytic mechanism. The functions and role of PTPs in the BBB are diverse and PTPs have multiple targets in the BBB. Research carried out on tyrosine phosphatases, suggest that they are inversely related to MMP activity and are involved in the regulation of BBB permeability [346-348]. Since then over 100 PTPs have been discovered and divided into four classes; Classes I-III are cysteine based and the final aspartate based. Class I is the largest and most abundant group, which is further subdivided into the classical and the dual-specific phosphatase (DSP). The classical family include both receptor and non-receptor subtypes. An increase in certain PTPs is also associated with diseases such as diabetes and Alzheimer's disease. Thus have been investigated as a potential therapeutic target [349-351]. A recent study carried out on molecules that mimic Pleiotrophin, a growth factor with a high affinity for heparin, inhibits the receptor protein tyrosine phosphatases (β/Z), which lead to a reduction in amphetamine induced toxicity, this group have suggested that targeting the inhibition of receptor protein tyrosine phosphatases may protect against other brain disorders such as drug addiction [352]. The inhibition of PTPN-2 also has a protective effect against brain disorders as it prevents the Ang-1 induced dephosphorylation of Occludin and maintains integrity of the BBB [353, 354]. As the classical family of PTPs consists of multiple members, which have multiple targets, some members of have dual roles associated with BBB integrity. SHP-2 dephosphorylates PECAM-1 resulting in the disassociation and the relocation of β-catenin to the adheren junctions [291].

The dual specific phosphatases dephosphorylate all types of amino acid targets (Tyr, Ser and Thr), the most common being MAPK phosphatase and PTEN. Induction of PTEN through the administration of Sunitinib results in the decrease

in PDGFR signalling and is associated with a reduction of medulloblastoma cell migration across the BBB [355]. Class II is a low molecular weight phosphatase represented by one gene ACP1 associated with the clinical manifestation of Diabetes. Class III contains only three members; Cdc25 with isoforms A, B and C. Cdc25 are involved in the cell cycle in particularly in the induction of mitotic progression. Cdc25 is engages with the Notch signalling pathway in the brain, both are crucial for the progression of endocycling and a potential target for the treatment of low-grade gliomas [351, 356]. The fourth group are the *Drosophila* eye-absent gene (EYA1-4) involved in the development of eye, kidney, branchial arches and the ear.

Vascular endothelial phosphatase tyrosine phosphatase (VE-PTP, PTPRB) is a classical receptor like phosphatase, which targets the tyrosine sites on VE-cadherin (previously mentioned in section 1.5.3). Its activity has a protective effect on the BBB [258, 261].

1.8.2 Serine/Threonine Protein Phosphatases

Serine/Threonine protein phosphatases (PSPs) were first discovered by Gerty and Carl Cori in 1945. Initially thought to be a prosthetic group removing enzyme and hence named PR. Further investigation unveiled this enzyme to be Protein Phosphatase-1. Since then approximately 30 Ser/Thr protein phosphatases have been identified and are characterised by their target subunits. There are currently three structurally related families;

1. The cysteine based phosphoprotein phosphatases (PPP) which consist of further subfamilies PP1, PP2A PP2B etc.,
2. The cysteine based metal (Mg^{2+}/Mn^{2+}) dependent phosphatases (PPM), containing only 2 subfamilies; PP2C and pyruvate dehydrogenase, finally
3. The aspartate protein phosphatases (FCP and SCP), which contains the C-terminal domain phosphatase targeting the RNA polymerase II.

There are over 400 Ser/Thr kinases, which is considerably more than the known Ser/Thr phosphatases. This dichotomy is counteracted by the formation of the trimeric Ser/Thr phosphatase holoenzyme, which comprises of shared scaffolding and catalytic subunits binding to multiple alternating regulatory subunits. The multiple regulatory subunits allow for multiple Ser/Thr phosphatase configurations and thus facilitating different substrate specificity [344, 357, 358].

Alternative to a regulatory subunit, PPMs contain conserved sequence motifs regulate substrate specificity. Both PPP and PPM are dependent on metal ions for catalytic activity through the activation of water molecules to facilitate dephosphorylation. In contrast the FCP utilises an aspartate-based dephosphorylation mechanism [344].

1.8.3 PP1

PP1 is a Ser/Thr phosphatase involved in a range of processes including meiosis, mitosis protein synthesis, cell metabolism, cell membrane and cytoskeleton rearrangement and apoptosis. This myriad of functions is facilitated by the alternating structural compositions of PP1 allowing for a stringent targeting of a wide range of specific substrates [359].

1.8.3.1 Structure

PP1 is a dimeric protein consisting of a catalytic and regulatory subunit. It interacts with over 200 target proteins. The catalytic subunit is a highly conserved protein sequence among eukaryotes. This 38kDa protein has four isoforms, transcribed from the genes PP1 α and PP1 β/δ, and splice variants PP1γ_1 and PP1γ_2 [360]. All ubiquitously expressed apart from the latter, which can only be found in the testis [361]. The catalytic subunit has a particular folding pattern to create an active site, which is common to all PP1s. X-ray crystallography analysis enabled the visualisation of the catalytic subunit, which consists of two α-helical structures enclosing a β-sheet domain creating compact α/β fold. The β-sheet contains the Mn^{2+} and Fe^{2+} binding domains located at the active site [361]. Binding of the metal ions is regulated by a combination of amino acids aspartic acid, asparagine and histidine, a characteristic common to all PPP family members. The α/β fold creates a Y shaped shallow catalytic grooves. Water molecules bind to the metal ions initiating activation on the phosphorous atom of the target protein [362].

Approximately 100 regulatory subunits identified which bind to the catalytic subunit [363]. The regulatory subunit has the ability to control cellular localisation and substrate specificity. This subunit also contains a conserved identifiable RVxF sequence, which lies close to the binding site [360, 364]. This motif creates two deep hydrophobic pockets common to all PP1and not PP2A or PP2B. Although this is the most commonly characterised binding site other studies. The first reported regulatory subunit PPP1R12A (formally MYPT1) in 2004 [362]. MYPT1 binds to multiple points on PP1 including the RVXF motif resulting in altering configuration of the catalytic domain and regulating substrate specificity [359]. PPP1R12A binds

to PPP1CB forming an extended acidic groove. This acidic groove increases the affinity to the basic N-terminal of the myosin regulatory light chain.

Multiple endogenous inhibitors; inhibitor-1, inhibitor-2, CPI-17 and DARPP-32 (dopamine- and cAMP-regulated phosphoprotein; molecular weight 32 kDA) regulate PP1 [365]. Many PPPs can also be inhibited by small molecular toxins, which bind to the active site. The most commonly documented include microcystin, nodularin, calyculin A, tautomycin and okadaic acid. These small inhibitory molecules are often used to determine target proteins.

Due to similarities of the binding site in the PPP family, especially PP1 and PP2A there are often overlapping activity of these small inhibitory toxins. OA inhibits both PP2A and PP1 but the IC_{50} for PP1 is larger than that of PP2A and hence why it is often used as an inhibitor for PP2A. Crystal structure analysis of OA bound to PP1/PP2A demonstrated the common binding of OA to the α7- α8 position however PP2A encloses OA increasing binding integrity, compared to PP1, which remains open. OA also binds and inhibits PP4 and PP5 with a larger $IC_{50.}$ OA binds to the active site, preventing phosphatase activity.

While other toxins PP2A and PP1 have similar IC_{50} values. PP2B has a 10^3 – 10^4 fold lower IC_{50} values. Considering the near identical active sites this reflects the complexity of PPP regulation and activity. One explanation is the slight sequence differences as demonstrated by alternating Cys^{273} to the Ala, Ser or Leu found in PP2B increases the IC_{50} values 10-20 fold [366, 367].

1.8.3.2 Role

As previously mentioned PP1 is involved in cellular metabolism however it also plays a crucial role in glycogen metabolism and regulation of blood glucose levels. Together with phosphorylase a PP1 acts as a glucose sensor. Phosphatase A binds to PP1 in its active state and prevents the activity of PP1. High glucose concentrations convert phosphorylase to its inactive 'T' state. This in turn

disassociates from PP1 to activate glycogen synthase. Glycogen synthase converts phosphorylase A to phosphorylase B which does not bind to PP1.

The PP1 subunit isoforms α, β, γ1, and γ2 are expressed in the brain tissue. The β isoform is more highly expressed in the cell soma whereas the γ1 is highly expressed in dendrites and presynaptic neurons where it colocalises with calcium/calmodulin-dependent protein kinase type II (CaMKII) and synaptophysin [368].

The PP1 inhibitor I1 is strongly expressed in the CA1 region of the hippocampus [369. I1 activity is increased by PKA phosphorylation. The inhibtion of PP1 by I1 results in the increase in CaMKII Thr286 phosphorylation in LTP. This also demonstrate that cAMP pathways utilises PP1 to gate CAMKII in LTP which occurs in the postsynaptic neuron {Blitzer, 1998 #3029, 370-372].

PP1 regulates AMPA receptors in neurons firstly through its localisation with spinophilin and secondly through DARPP-32, which when phosphorylated inhibits PP1 associated with increased AMPA receptor currents [373]. Spinophilin KO mice have been associated with reduced brain size, altered AMPA and NMDA receptor functions and reduced long-term depression. While, also displaying a higher resistance to kainite-induced seizures [374]. The AMPA receptor channel phosphorylation occurs as Ser^{831} and Ser^{84}. PKA regulates Ser^{845} phosphorylation enhancing channel activity during LTP [374]. CaMKII phosphorylates the Ser^{831} whilst also increasing channel activity however this does not occur in all neurons. During LTD PP2B dephosphorylates I1, which inturn increases PP1's dephosphorylation of AMPA at the Ser^{845} inhibiting channel activity. In addition PP1 and PP2B have been associated with AMPA endocytosis during LTD upon dephosphorylation of the Ser^{845} [375-377]. PP2B also regulates the NMDA receptor through DARPP-32 and the direct dephosphorylation of NMDA receptor decreases channel opening [378].

PP1 has also been linked to long lasting, repetition learning. The use of the Morris water maze test and the drug-awarding test determined that PP1 inhibition with I1

and DARPP-32 lead to attenuation in memory retention. Mice with inhibited PP1 required shorter intervals between training episodes in order to adequately complete the course [379-381].

PP1 in the brain also has a role in regulating the circadian clock, PP1 inhibition alters the light induced resetting of the circadian clock in mice [382]. The period gene (PER) has multiple phosphorylation sites, 24 of which are regulated by PP1 and PP2A. In particular mutation of the Thr^{610} and Ser^{613} restored circadian rhythm, however this extended the cycle to 30 h. This may be due to phosphorylated Ser^{613} induced regulation of the phosphorylation site Ser^{586} [383, 384]. PP1 is also required to maintain PER expression as loss of PP1 results in PER degradation [385]. PP1 has also been shown to regulate the timeless gene (TIM) however there is less known about its regulation [384, 386]

1.8.3.3 Disease

PP1 has been associated with many neurological disorders such as Parkinson's, Alzheimer's and Huntington's disease. The LRRK2 gene is the largest contributor to genetically linked Parkinson's disease. PP1 forms a complex with LRRK2 and dephosphorylates multiple Serine sites (Ser^{910}, Ser^{935}, Ser^{955} and Ser^{973}). This phosphorylation is associated with the manifestation of Parkinson's disease [387-389]. Conversely, spinophillin is a major PP1 targeting protein, which has been linked to the decrease in PP1 seen in Parkinson's diseased animal models [390].

A role of PP1 in the manifestation of Alzheimer's disease has been discovered. Early studies reported a decrease in PP1 activity in both white and grey matter in AD patients [391]. Alzheimer's disease is also characterised with an accumulation of amyloid β, tau and microtubule hyperphosphorylation [392-394]. The amyloid β precursor protein (APP) is hyperphosphorylated at Thr^{668} in the Alzheimer's diseased brain by GSK-3β and Cdk5. PP1 and PP2A are associated with the dephosphorylation of APP Thr^{668} [393]. More recently PP1 has been shown to form a complex with the APP and resulting in dephosphorylation (Thr^{668}) [395]. Accumulation of APP also has an additional inhibitory effect on PP1 contributing to the manifestation of Alzheimer's disease [396]. Dopamine- and cAMP

phosphoprotein 32 kDa (DARPP-32) is a PP1 inhibitor that regulates CREB phosphorylation. DARPP-32 is cleaved at Thr^{153} by calpain inducing PP1 inhibition associated with Alzheimer's disease and amyloid-beta treated neuronal cells [397]. APP induces acute depression of the excitatory neurons and PP1/PP2A have been associated with this induction. PP2B has also been associated with the APP induced depression however the roles of these phosphatases differ and do not overlap [394].

Increased phosphorylation of tau in Alzheimer's disease is associated with loss of PP1 [398]. Overexpression and knockout studies of Egr-1 in rat brain confirmed the role of PP1 in tau phosphorylation. Erg-1 both activates Cdk5 and inactivates PP1, increasing tau phosphorylation at $Ser^{396/404}$ and Ser^{262}, which in turn destabilises the microtubules [399]

The association between PP1 and Huntington's disease remains to be fully elucidated. However what remains constant is the preventative effect of PP1 inhibition, preventing neuronal cell death in Huntington's disease models [400-402]

1.8.4 PP2B

PP2B is ubiquitously expressed Ser/Thr phosphatase also known as calcineurin. It is a highly conserved protein in eukaryotes. Its activity is calcium dependent and exists as a heterodimer consisting of the ~60 kDa catalytic and ~20 kDa regulatory subunits. The discovery of PP2B was due to its inhibitory effect on calmodulin-dependent cyclic nucleotide phosphodiesterase.

1.8.4.1 Structure

Activation of PP2B involves the binding of calcium to the regulatory subunit, which induces structural alterations allowing the binding of calmodulin to the α-helix of the catalytic subunit. This removes an autoinhibitory helical structure from PP2B active site. PP2B's active site is similar to the PP1 active site previously discussed. The PP2B family contains three catalytic isoforms (α, β, and γ) than two regulatory subunits (type 1 and 2). In contrast to PP1, the PP2B family of isoforms also lacks

functional and structural diversity. PP2B interactions are facilitated through two points on the surface. The first is a β-sheet on the catalytic subunit, which interacts with the conserved PxIxIT motif. The interaction forms an extended β-sheet. This motif recruits nearby substrates. Many proteins which interact with PP2B contains the PxIxIT motif such as the nuclear factor of activated T-cells, scaffolding protein regulator of calcineurin, myosin phosphatase and dynamin 1. The second motif is the LxVP found near to the interface of the catalytic and regulatory subunits, binding to both when activated. This motif is also found on nuclear factor of activated T-cells and TRESK and PKA-RII α.

1.8.4.2 Role

PP2B is abundantly expressed in the brain along with other non-neuronal tissue. PP2B is involved in multiple regulatory processes in the body such as mitosis, meiosis, cell metabolism, synaptic plasticity, insulin signalling and cellular apoptosis. PP2B has a well-documented important role in the immune response with regard to the regulation of transcriptional processes [403]. PP2B is a therapeutic target for the immunosuppressant's FK506 and cyclosporin to treat organ transplant and rheumatoid arthritis. Cyclosporin binds to PP2B and prevents binding of the PxIxIT motif of NFAT and in turn its dephosphorylation. Phosphorylated NFAT supresses T-cell activation. Cyclosporin and FK506 have also been shown to have a neuroprotective effect against ischemia in the rat forebrain by the inhibition of PP2B [404].

In the brain, PP2B has a vital role in the regulation of neuronal structural plasticity, long-term potentiation (LTP) and long-term depression (LTD) [405, 406]. The induction of LTP has been extensively investigated and involves many Ser/Thr phosphatases. Inhibition of PP1, PP2A and PP2B by multiple molecules all prevent LTP induction [407-410]

A positive feedback loop is involved in the switching from LTP and LTD. This involves PP2A dephosphorylation of DARPP-32 (Thr^{75}). A controlling factor of this pathway is the intercellular availability of Ca^{2+}, which controls PP2B activity [411]. Strong signals stimulate a high influx of Na^{2+} through the glutamate AMPAR, this

displaces Mg^{2+} for the NMDA channel facilitating the influx of Na^{2+} and Ca^{2+}. High levels of intercellular Ca^{2+} binds to PKA and C. These kinases phosphorylate cytosolic AMPAR, transporting it to the membrane. Low pre-synaptic signals and reduced intercellular Ca^{2+} results in the activation of PP2B as it has a higher affinity to Ca^{2+}[412].

Subsequently AKAP150-anchored PP2B removes the phosphatase from AMPAR resulting in decreased activity and membrane expression of AMPAR during LTD [413]. PP2B is required for the inhibition of LTP and the inhibition of PP2B prevents LTD and synapse attenuation [414, 415]. It has been hypothesized that inhibiting PP2B and its effect on LTD can prevent memories associated with fear [416]. Nicotine has been shown to prevent the consolidation of LTP. However PP1/2A/2B inhibitors all prevent the nicotine-induced LTP reversal indicating that each phosphatase is involved [417]. In diabetic rats the inhibition of PP2B prevented LTD induction without effecting LTP [418].

Small inhibitory molecules for PP2B include the INCA compounds and VIVIT peptide. Both target the PxIxIT motif-binding site inducing dissociation. This is a more specific alternative to inhibiting the active site as this would have off target effects [419].

1.8.4.3 Disease

Considering the role of PP2B in memory and learning, PP2B has been associated with Alzheimer's disease. Aβ induces dysfunction in both pre and postsynaptic neurons in the brain [415]. Inhibition of PP2B with FK506 has previously been shown to reverse this effect whereas inhibition of PP1/PP2A attenuated the effect of Aβ in postsynaptic neurons alone. Signifying the multifunctional but diverse roles of PPPs in the Aβ induced depression of the brain neuronal synapses [394]. In the postsynaptic neurons Aβ increases NMDA receptor endocytosis through PP2B activity [420].

The abundance and activity of PP2B in an Alzheimer's diseased brain has been debated over a number of years. Some have immunoprecipitated PP2B from diseased brain samples and after measuring activity reported a three-fold increase in PP2B activity (activation due to calpain I cleavage of PP2B)[421]. Others have selectively inhibited PP2B with cyclosporin A, this did not affect tau phosphorylation, accumulation or function. Alternatively suggesting PP2A as the phosphatase involved in tau phosphorylation associated with Alzheimer's disease [422].

However, Calcipressin1 is an inhibitor of PP2B, which has been shown to increase during Alzheimer's diseased and aging brains [423]. Other groups have demonstrated a decrease in PP2B activity but not expression in human Alzheimer's diseased brain samples [424, 425]. While previous studies have shown decrease in tau phosphatase activity in Alzheimer's disease and PP2B having an inverse relationship with tau phosphorylation [426-428]. PP2B and PP2A dephosphorylate tau at $Ser^{199/202}$ and partially at $Ser^{396/404}$. Additionally, the Ser^{235} is dephosphorylated by PP2B alone [429, 430]. PP2B along with PP2A and PP1 in vitro treatment of an Alzheimer's disease models have the ability to dephosphorylate tau and restored normal microtubule assembly [426, 428, 431, 432]

This disparity in PP2B expression may be due to altering expression in different locations in the brain. PP2A and PP2B increased expression have been shown in astrocytes of the Alzheimer's diseased temporal cortex [433]. Inhibition of PP2B by neuroleptics has also been suggested as a contributed to phosphorylated tau in Alzheimer's disease [434]. Additionally, PP2B has been associated with neuronal death. In healthy brain tissue LpE inhibits PP2B activity while also preventing oxidative stress induced cell death. In a diseased state, LpE is lost; PP2B is activated associated with neuronal cell death [435].

1.8.5 PP2A

PP2A is highly conserved and ubiquitously expressed enzyme. Eukaryotes share a >78% amino acid sequence homology [436]. Due to its multimeric composition PP2A accounts for 1% of the total cellular protein [437, 438]. Facilitating its multifaceted role in the cell. PP2A has an essential role in the majority of cellular maintenance and development such as cell cycle regulation, DNA replication, transcription, translation, cell morphology, motility, proliferation, signal transduction and apoptosis [439-444]. The importance of PP2A activity is evident as early as embryological development. Mouse and drosophila knock out studies have demonstrated that a functioning PP2A catalytic subunit is necessary for the developing embryo [441, 442].

1.8.5.1 Structure

The structure of PP2A allows for its multiplicity. PP2A functions as a complex trimeric protein complex consisting of a catalytic subunit (C), a scaffold subunit (A), and one of the alternative regulatory subunits (B; Figure 1.8). In mammals, α and β isoforms exist for both the catalytic and scaffolding subunits [441]. There are four B subunit families, each with several isoforms or splice variants (Table 1.1). Considering there are 2.85 times more Serine/threonine kinases, the multimeric form of PP2A and diversity of these subunits allows for the large number of substrate specificities [344, 445, 446].

Previous reports also suggest the PP2A can exist as a dimer composed of just the catalytic and regulatory subunit [439, 447]

1.8.5.2 Scaffolding Subunit

The scaffolding subunit is a 65kDa protein, also known as the PR65, which facilitates the structural assembly of PP2A. Acting as a support for catalytic and regulatory subunit binding. It has two isoforms, PR65α and PR65β, which are encoded by PPP2R1A and PPP2R1B respectively [448, 449]. Both are ubiquitously expressed and share an 86% amino acid sequence homology [450, 451]. PR65α is the predominant isoform found in 90% of PP2A holoenzyme assemblies. Both isoforms differ in their ability to bind to the different regulatory subunits and are a determining factor [452].

PR65 contains a two-layered α-α solenoid Huntington/elongation/A-subunit/PI3 kinase target of rapamycin 1 (HEAT) sequence [453]. The HEAT sequence is 39 amino acids in length and repeats 15 times in tandem, enables protein binding and supports the holoenzyme structural integrity [454, 455]. The α helices are linked through a tight horseshoe 1-3 residue turn [451]. The regulatory subunit binds to the HEAT sequence at points 1-10 followed by the catalytic subunit binding to the point 11-15 through a combination of hydrogen bonding, hydrophobic and ionic interactions [449, 456-458]. The scaffolding subunit is a flexible structure bending to expose the active site of the catalytic subunit, enable binding of catalytic and regulatory subunits and recruitment of the regulatory subunit, which is facilitated by the HEAT sequence repeats [455].

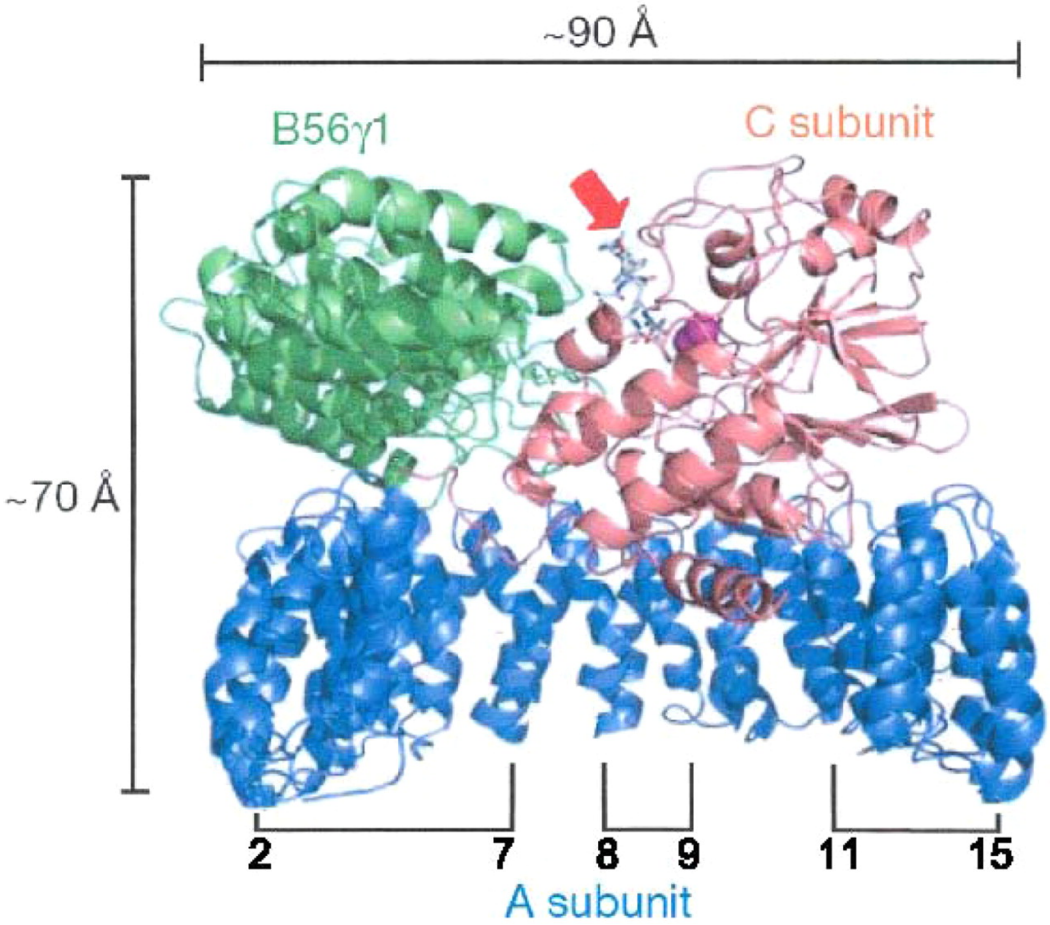

Figure 1.8 Crystal structure of PP2A.

Crytal representation of a PP2A heterotrimeric holoenzyme, the scaffolding subunit (A, blue), regulatory subunit (B, green) and catalytic subunit (C, red). The red arrow highlights the active site.

Taken from Phillips 2009 [459].

1.8.5.3 Catalytic Subunit

The two members of the PP2A catalytic subunit family are PP2Acα and the PP2Acβ encoded by the PPP2CA and PPP2CB genes respectively. Both are 37 kDa in size and have 97% amino acid sequence identity. PP2Acα accounts for the majority of PP2A activity and is more abundantly expressed than PP2Acβ. This is due to a stronger promoter activity and more efficient mRNA turnover [460, 461]. Due to their similarity, the structural formation of PP2A is not dependent on the catalytic subunit [462]. The PP2Ac subunit is highly conserved as the human form shares close it 90% sequence homology with the form found in yeast [436]. Due to the evolutionary relationship between PP2A and PP1 (Figure 1.9) they have a 50% amino acid sequence homology [446].

PP2Ac expression undergoes both translational regulation and post-translational modifications [463]. Although PP2Ac shares a high homology with PP1, PP2B, PP4 and PP6, PP2A contains a unique TPDYEY motif in the C-terminal tail [449, 457]. This motif undergoes both phosphorylation and methylation, which alters enzyme assembly and activity [464, 465]. Reversible phosphorylation of Tyr^{307} through GSK3β and v-Src kinase activity and prevents holoenzyme assembly and inhibits activity [466, 467]. Leu^{309} is also reversibly modified however through methylation [468]. Opposing the phosphorylation of the TPDYEY motif, Leu^{309} methylation results in the increase in PP2Ac activity as it promotes assembly but also determines subunit composition [469, 470].

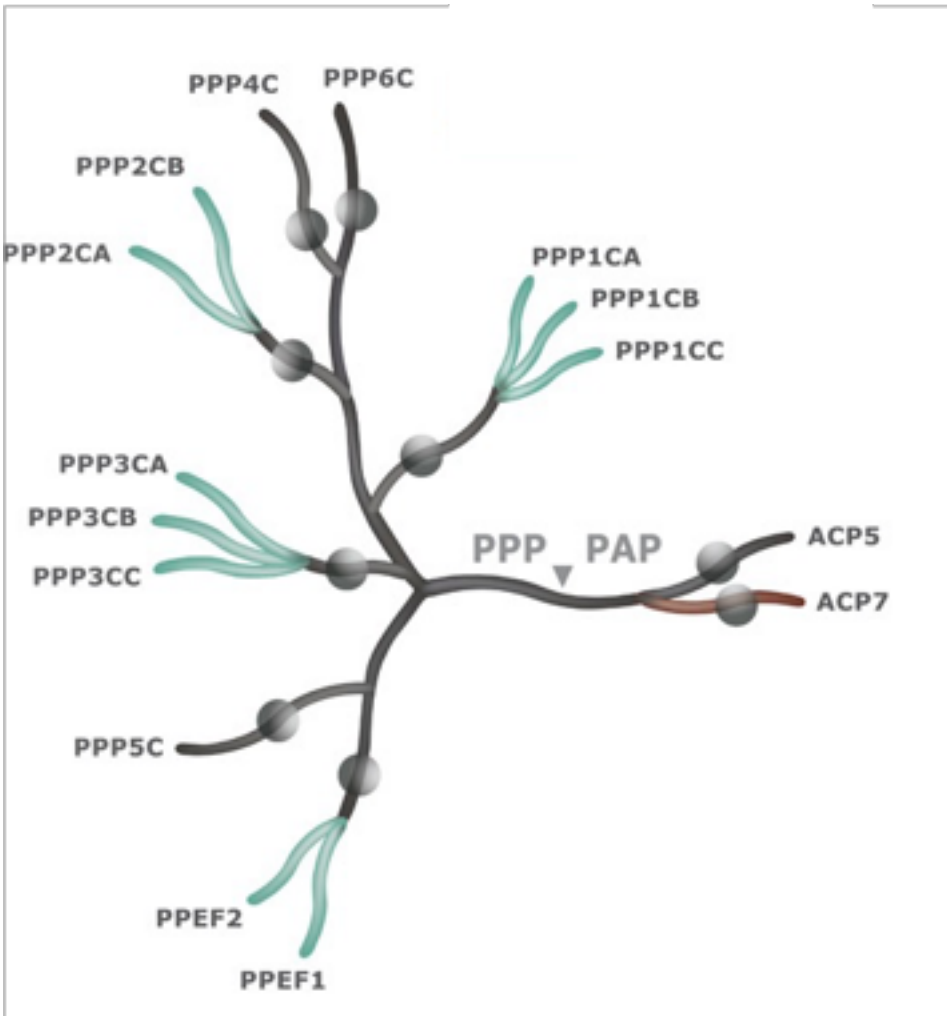

Figure 1.9 Protein Phosphatase phylogenetic tree.

Taken from Chen *et al* 2017 [446].

1.8.5.4 Regulatory subunit

The regulatory subunit of PP2A is the main contributor to the diversity and complexity of PP2A. There are 15 genes encoding these subunits, however the combination of transcriptional and splice variants results in close to 30 different isoforms in the regulatory subunit family ranging from 55-130kDa that have no sequence uniformities [456, 471]. The isoforms are classified into four families:

B

This family is also known as B55 (B subunit, 55kDa) and consists of 4 isoforms that share a high portion of sequence similarities (α, β, γ and δ) [472]. The isoforms differ in their expression and subcellular localisation [441]. The B55α and B55β isoforms bind to over 100 proteins. B55α and B55δ are expressed in majority of cells whereas B55β and B55γ are localised in the brain [473]. B55 is involved in a multitude of cellular functions such as DNA replication and repair, cellular division, signalling and metabolism, cytoskeleton regulation, mitochondrial function and molecular trafficking [474]. The B55 family has been of particular interest in the area of cancer research and as a potential tumour suppressor. The loss of B55 has been linked to prostate cancer, breast cancer, colorectal cancer, lung cancer, leukaemia and teratomas [475-479].

B'

Also known as the B56 subunit, is the most abundant of the regulatory subunits. B56 (B subunit, 56kDa) consists of 5 isoforms (α, β, γ, δ and ε) and unlike other families undergoes phosphorylation [480]. B56 has a prominent role in apoptosis through the regulation of molecules involved in apoptosis such as P53, BCL2, MYC, GSK3 and the extracellular receptor kinases [442, 481-485]. B56 is also involved in cell cycle check-points, growth factor and Wnt signalling [486-489].

B''

The penultimate family of regulatory subunits is made up of both 72kDa (PR72) and 130kDa (PR130) proteins, which is highly expressed in heart and skeletal muscle [439]. The former is uniquely found in heart and skeletal muscle and the

latter is ubiquitously expressed [490]. In comparison to the B55 and B56 families, less is known about the PR72/PR130 family of subunits, which appear to be evolutionary divergent [491]. These subunits contain a calcium-binding motif, which modulates the PP2A holoenzyme activity [490, 492]. The PR72 regulates the DARPP-32 in the Brain [492]. PR72/PR130 has previously been shown to be involved in the maintenance of DNA integrity, stem cell pluripotency, regulation of the WNT/β-catenin and EGFR signalling [493-495].

B’’’

The last regulatory subunit family include striatins, of which there are 3 (STRN, STRN3 and STRN4) and serine/threonine-protein phosphatase 2A activator (PTPA) [496, 497]. These are calmodulin binding proteins, the binding of calmodulin to PP2A enhances Ca^{2+} dependent signalling [441]. Stratins are found in complexes composed of PP2A and kinases such as Germinal Centre Kinase III and the STRN interacting phosphatase and kinase (STRIPAK) complex [498-500]

Name	Alternative Name	Gene	Chromosome Location
Scaffolding Subunit (A)			
PP2A-Aα	PR65α	PPP2R1A	19q13.41
PP2A-Aβ	PR65β	PPP2R1B	11q23.2
Regulatory Subunit (B)			
PP2A-Bα	B55α	PPP2R2A	8p21.2
PP2A-Bβ	B55β	PPP2R2B	5q32
PP2A-Bγ	B55γ	PPP2R2C	4p16
PP2A-Bδ	B55δ	PPP2R2D	10q26
PP2A-B'α	B56α	PPP2R5A	1q32.2
PP2A-B'β	B56β	PPP2R5B	11q13
PP2A-B'γ	B56γ	PPP2R5C	14q32
PP2A-B'δ	B56δ	PPP2R5D	6p21
PP2A-B'ε	B56ε	PPP2R5E	14q23
PP2A-B''α	PR72/PR130	PPP2R3A	3q22.1
PP2A-B''β	PR48	PPP2R3B	37p13
PP2A-B''γ	PR59	PPP2R3C	14q13.2
Striatin		STRN1	2p22
Striatin 3		STRN3	14q12
Striatin 4		STRN4	19q13
Phosphotyrosyl Phosphatase Activator	PR53	PPP2R4	9q34.11
Catalytic Subunit (C)			
PP2A-Cα		PPP2CA	5q31.1
PP2A-Cβ		PPP2CB	8p12

Figure 1.1 List of the various subunits of PP2A

1.8.5.5 Function

The PP2A dimeric or trimeric holoenzyme has multiple combinations allowing it to dephosphorylate over 300 substrates and is key component in numerous signalling pathways involving WNT, PI3K/Akt, MAPK and mTORC [501-503]. Its complexity enables PP2A to have a dual role in signalling. For example, over expression of PP2A-Cα activates WNT signalling, while overexpression of the scaffolding subunit PP2A-B'ε suppresses WNT signalling [504].

1.8.5.6 Pre and Post-translational Regulation of PP2A

PP2A expression and activity can be regulated both pre and post-translationally. Different miRNAs target multiple PP2A subunits, preventing translation. Post-translational modifications of PP2A include methylation, nitrosylation and phosphorylation, which block the assembly and in turn activity of PP2A.

1.8.5.6.1 miRNA

miRNAs regulate and inhibit the translation of ~60% of mammalian proteins including the PPP family of phosphastases [505]. miR-1 and miR-133 prevent the translation of PPP2R5A and PPP2R5D respectively in the heart. Resulting in a decrease in PP2A activity and increased phosphorylation of the ryanodine receptor and arrhythmogenesis [506, 507]. miR-133b also targets PPP2R5D expression in hepatocellular carcinoma and is associated with an increase in drug responsiveness [508]. However other miRNA's have been associated with a decrease in drug responsiveness such as the miRNA200c, which attenuates PPP2R2B expression in oesophageal cancer [509]. miR-652 targets the PPP2R3A, which induces epithelial-mesenchymal transition in prostate cancer [510]. miR-183, miRNA17-92, miR-587 and miR-429 all target the PP2A catalytic subunits in renal cancer, lymphoma, colorectal cancer and osteoblastic cells respectively [511-513].

1.8.5.6.2 Phosphorylation

As previously mentioned in Section 1.8.5.3, the catalytic subunit of PP2A contains a TPDYEY motif in the C-terminal tail, which undergoes phosphorylation. Tyr^{307}

phosphorylation attenuates PP2Ac activity by attenuating regulatory subunit binding to the catalytic and scaffolding subunit, thus inhibiting holoenzyme assembly [464, 514, 515]. Tyr^{307} phosphorylation enables CIP2A and SET binding while also preventing serine/threonine-protein phosphatase 2A activator binding, which contributes to the inhibitory effect [516-518]. An Alzheimer's disease study suggests that the phosphorylation of Tyr^{307} effects the Leu^{309} methylation discussed below [519]. Further investigations into the TPDYEY motif unveiled the Thr^{304} residue phosphorylation, which also prevents subunit binding and holoenzyme assembly [464, 514, 520, 521]. GSK3β and v-Src are two kinases associated with Tyr^{307} phosphorylation [466, 467]. PTPA has also been associated with the dephosphorylation of the Tyr^{307} residue in rat hippocampus [522]. Less is known about Thr^{304} phosphorylation regulation.

The regulatory subunits of PP2A also undergo phosphorylation. Extracellular signalling regulated kinase (ERK) targets the Ser/Pro residue on B56, increase in the phosphorylation B56 prevents binding to the PP2Ac [449, 481]. Interestingly, phosphorylation of the regulatory subunits B56α, B56δ and B'' family results in the increase in PP2A activity and enzymatic efficiency. The former phosphorylated by protein kinase R and the latter two by protein kinase A. Resulting in the increase in PP2A activity and enzymatic efficiency [523-525].

1.8.5.6.3 Methylation

The TPDYEY motif also undergoes methylation (as discussed in Section 1.8.5.3). Transient alterations in PP2Ac methylation are highly involved in cell cycle regulation [526, 527]. The Leu^{309} residue is methylated by the leucine carboxyl methyltransferase 1 (LCMT-1) [528, 529]. The increase in methylation promotes PP2A holoenzyme assembly through enhanced binding of the catalytic subunit to the regulatory subunits [469, 514], while also increasing PP2A activity [530]. Loss of LCMT-1 and associated demethylated PP2Ac has been detected in clinical samples of Alzheimer's disease [531]. The action of LCMT-1 is reversed by PP2Ac methylesterase-1 (PME-1). PME-1 acts by directly binding to PP2A and removes the Mn^{2+} ions from the active site, attenuating PP2A activity [532, 533]. PME-1

knockout mice do not survive 24 h after birth, demonstrating the vital role of PME-1 for survival [534]. PTPA has the ability to reverse PME-1 binding to increase PP2A activity. PTPA also increases PP2A activity through the induction of a PP2A conformational change enabling binding of the catalytic and regulatory subunits [533, 535].

1.8.5.6.4 Nitrosylation

Although it is less understood as the previous modifications. PP2A also undergoes nitrosylation, which is suggested to counteract the inhibitory effect of phosphorylation resulting in the increase in PP2A activity. Nitric oxide synthase targets the Tyr^{307} residue for nitrosylation [521, 536]

1.8.5.7 PP2A inhibitors

PP2A activity is modulated through the action of 2 endogenous inhibitors, CIP2A and SET. Inhibition of PP2A shifts the balance towards kinase activity [537] and hyper-phosphorylation which is detrimental to the cells homeostatic regulation and function [538]. Interestingly, hyper-phosphorylation and over expression of CIP2A is frequently found in diseases such as cancer [445, 539].

1.8.5.7.1 Alpha 4

Alpha 4 (α4; also known as mTAP42) is a 52 kDa alpha helical protein which is widely expressed [540]. It has been shown to act as a scaffold for PP2Ac and Mid1 [541]. α4 and the PP2A scaffolding subunit have overlapping binding sites on PP2Ac and thus binding of α4 displaces and prevents binding of the scaffolding subunit [542]. The binding of α4 to PP2Ac also stabilises the holoenzyme structure under cellular stress conditions and protects it from proteosomal degradation. Loss of α4 results in the ubiquitination and proteosomal degradaiton of PP2A [543, 544]

1.8.5.7.2 $I1^{PP2A}$

Inhibitor 1 of PP2A ($I1^{PP2A}$, also known as pp32 or acidic leucine-rich nuclear phosphoprotein 32 member A (ANP32A)) is a sphingosine sensitive inhibitor of PP2A through the binding to catalytic subunit [545, 546]. $I1^{PP2A}$'s regulation of PP2A has been implicated in a multitude of cellular functions such as signal transduction, cytotoxicity and tumour suppression [547] [548, 549]. An isoform known as ANP32e also has an inhibitory effect on PP2A and is involved in synaptogenesis and neuronal development [550, 551].

1.8.5.7.3 CIP2A

CIP2A (cancerous inhibitor of PP2A) is a 90kDa oncoprotein previously known as kIAA1524. Its expression is found in 39-90% of cancers and is associated with poor prognosis [552-554]. CIP2A is an endogenous inhibitor of PP2A through the direct binding to prevent activity. CIP2A is mainly located in the cytoplasm of cancer cells but is also found expressed diffusely in the tumour mass [555]. CIP2A is involved in many tumour characteristics such as stimulating anchor dependent cell growth, progenitor cell renewal, cell cycle progression and mitosis [556, 557]. PIK1 is a protein required for cell cycle progression, CIP2A binds to PIK1, preventing ubiquitination and increasing stability [558]. CIP2A also increases tumour growth through the activation of c-MYC, preventing the inhibitory effect of PP2A [559]. Autophagy is also prevented by CIP2A. Autophagy is inhibited by MTORC-1, whose key regulator is CIP2A [560-562]. CIP2A not only decreases PP2A phosphatase activity but also increases Ser/Thr kinases activity, AKT in HCC and breast cancer [563, 564]. Inhibition of CIP2A with siRNA results in the decrease in xenograft tumour growth [445], similarly miR-218 and mir-375 also prevent CIP2A transcription and are being investigated as a potential therapeutic target [565, 566]. The exact binding of CIP2A to PP2A remains unknown and the mechanism of inhibition poorly understood. However, it is suspected that CIP2A prevents the assembly of the holoenzyme through the interaction between the scaffolding and catalytic subunits [445].

1.8.5.7.4 SET protein

SET, also known as inhibitor 2 for PP2A (I^2PP2A), is an endogenous inhibitor of PP2A. The two isoforms of the inhibitors for PP2A (I^1PP2A and I^2PP2A) were first isolated from bovine kidneys and reported to have a potent inhibitory effect on PP2A activity [567]. The SET family include 2 splice variants, SETα and SETβ. Both contain an acidic C-terminal domain, nucleosome assembly protein domain and an N-terminal, which includes two serine phosphorylation sites. The acidic C-terminal mediates the direct binding to PP2A, phosphorylating PP2A at Thr^{307} of the catalytic subunit, which inhibits its activity [568-571]. Through this mechanism, SET increases its association with the activation site. Resulting in the increase in proliferative and oncogenic signalling such as Akt, Erk and c-Myc. SET expression is linked to multiple cancers [555, 570, 572, 573]. Resulting in the increase in cancer cell survival and tumour progression [517, 571, 574-576]. SET expression in particular has been associated with drug resistance in cancer. A study carried out on multiple colon cancer cell lines determined that the increase in SET resulted in drug resistance [577], while another study also demonstrated that SET over-expression was associated with paclitaxel resistance and reversed by SET silencing [569]. OP449 is a SET antagonist operating by directly binding to SET and preventing its interaction with PP2A. OP449 has been shown to successfully prevent drug resistance in leukaemia [578].

SET inhibits PP2A selectively at low abundance [579]. PI3K phosphorylates Ser^{93} and protein kinase C phosphorylates SET at Ser^{9} and Ser^{24}, enable binding to PP2A [580, 581]. However phosphorylation of Ser^{171} by protein kinase D attenuates SET mediated inhibition of PP2A [582]. SET induced inhibition of PP2A is also attenuated by the PP2A activators ceramide and FTY-720 (a sphingosine analogue). Both proteins prevent binding of SET to PP2A [579, 583].

1.8.5.8 Disease/PP2A in the brain

All of the known Ser/Thr phosphatases are detectable in the brain. The activity of PP2A is 3 times higher than that of the second most common phosphatase PP1 [439, 584, 585]. A decrease in PP2A activity is associated with epileptic seizures as a direct consequence of phosphorylation of tau [586] or due to inhibition of PP2A increasing zinc release and concomitant phosphorylation of tau in the synapse [587]. This is supported by further studies showing that sodium selenite, which increases PP2A activity reduces hyper-phosphorylation of tau and decreases seizures in rodent models of epilepsy [588].

Inhibition of PP2A cause an increase of phosphorylated APP, resulting in increased neurodegeneration [589]. Aging is linked with reduced drainage of the soluble β-Amyloid from the brain. The Aβ is formed by the severing of the Amyloid Precursor Protein (APP) by the protein secretases β-secretase (BACE-1) and γ-secretase (presenilin, PS-1/PS-23) which sever the N-terminal and C-terminal respectively. Research has shown that the inhibition in PP2A by okadaic acid results in an increase in enzymatic activity of β and γ secretase activity with a direct increase in phosphorylation at the serine APP^{668} site and in turn an increase in Aβ generation [590]. More recently, reduced PP2A activity has been linked to the progression of Parkinson's disease, due to increased phosphorylation of α-synuclein. Increased phosphorylation causes it to misfold and polymerise to form fibrils, a hallmark of Parkinson's and dementia with Lewy bodies [591].

PP2A has also been associated with the hyperphosphorylation of tau in Alzheimer's disease. The inhibition/downregulation of phosphatases in the brain hyperphosphorylates tau, preventing its binding to the microtubules. This results in aggregation in neurofibrillary tangles and dystrophic neuritis [592]. GSK-3β is a tau phosphorylating kinase. Inhibition of PP2A with okadaic acid has a dose dependent increase in tau phosphorylation while also activating mitogen-activated protein kinases ERK1/2 and MEK1/2 also associated with alzheimer's disease [593, 594]. PP2A specifically phosphorylates tau Ser^{202}/Thr^{205} and Ser^{46} in

response to microtubule depolymerization [595]. As previously mentioned Both PP2A and PP2B have been shown to dephosphorylate tau at $Ser^{199/202}$ and Ser^{396}/Ser^{404} [429, 430]. However, the involvement of PP2B in tau phosphorylation is still debated with multiple studies inhibiting PP2B determined that PP2B does not alter tau phosphorylation [422].

Loss of PP2A expression in the brain has also been detected in individuals with down syndrome and intellectual disability [596]. Hyperphosphorylation (Ser^{199}, Thr^{205}, Thr^{212}, Ser^{262}, Ser^{396} and Ser^{422}) and accumulation of tau associated with the loss of PP2A have also been linked to these individuals. Similar to Alzheimer's disease the down syndrome brain develop neurofibrillary tangles however at an earlier age (>30 yrs) [597].

1.8.5.9 Role of PP2A in the immune system

PP2A plays a role in the dysregulation of the immune system and inflammatory signalling [568, 598, 599]. The role of PP2A in T-cell function is seen in systemic lupus erythematosus (SLE), an autoimmune disease characterised by over-activation of the inflammatory response. In these patients, there is increased expression of PP2A in T-cells [600, 601]. PP2A also prevents the transcription of IL-2 by dephosphorylating the cAMP response element binding protein, preventing its interaction with the IL-2 promoter [602]. An induced overexpression of PP2A in T cells does not affect the baseline immune function, but exposure to a toxic serum leads to over-activation of the immune response characterised by increased production of neutrophils and IL-17 [600].

PP2A is also involved in macrophage maturation. Exposure of monocytes to colony stimulation factor 1 (CSF-1), an established factor used in macrophage maturation, increases PP2A expression [603]. However, in mature macrophages LPS reduces expression of IL-1 and IL-8 through a down regulation of TNF-α mediated through PP2A [598]. The association between PP2A and TNF-α signalling is confirmed using the plasma protease inhibitor, α_1-Antitrypsin (A1AT), which increases the activity of PP2A and prevents the inflammatory response triggered by TNF-α

[604]. A similar effect was seen in lung epithelial cells, using the PP2A activator FTY-720, which inhibited the production of IL-8 and IL-6 [605]. FTY-720 is more commonly known for its use in the treatment of multiple sclerosis, where it inhibits production of IL-2 and IFN-γ in $CD8^+$ T cells [606]. Together, these data indicate that stimulating PP2A could have therapeutic potential in diseases with an underlying inflammatory component. However, the role of PP2A in mediating blood brain permeability remains to be established.

FTY-720 is a stimulator of PP2A, known for its anti-inflammatory effects and currently used in the treatment of multiple sclerosis. Previous studies have demonstrated its protection against retinal barrier breakdown [607] and pulmonary endothelial disruption [608]. While FTY-720 analogues have also been shown to prevent TNF-α and LPS mediated increase in endothelial cell permeability [609]. However, this protective effect may be dose dependent [610].

1.9 Summary

The overall scope of this thesis is to investigate the association between PP2A and VE-cadherin abundance in brain microvascular endothelial cells. In particular, it focuses on how PP2A modulation may affect VE-cadherin abundance, the associated effect on endothelial permeability and whether this mechanism is involved in inflammation.

The "Nun Study" is a project carried out by David Snowden on the Sisters of Notre Dame, which found that the health of the brain microvasculature is a major determining factor to aging, memory retention and manifestation of dementia [611]. VE-cadherin is an integral component of the functioning endothelium in the blood brain barrier. Its transcellular abundance can be altered by post-translational modifications and hence why it is important we understand the mechanism of VE-cadherin regulation. PP2A is the most abundant phosphatase in the brain and its loss is associated with neurological diseases such as Alzheimer's disease. What remain undetermined is the role PP2A has on VE-cadherin in brain microvascular endothelial cells and vascular permeability. Using pharmacological modulators of PP2A, OA and FTY-720, I investigated how modulation of PP2A and any associated effect on the abundance of VE-cadherin. As the specificity of OA to inhibit PP2A is debated, I aim to use CIP2A and SET to confirm any effects of PP2A inhibition. The main function of VE-cadherin is the regulation of paracellular permeability. I plan to utilise a FITC-labelled dextran to evaluate paracellular permeability in order to investigate any physiologically relevant consequences of PP2A modulation on brain endothelial permeability.

Macrophages have both protective and damaging effects on the integrity of the brain microvascular integrity. Pro-inflammation is a known inducer of paracellular permeability, through the loss of VE-cadherin cell-cell interactions. An association that has yet to be investigated is the effect of macrophages on PP2A in the brain microvascular endothelial cell. Using a co-culture model of brain microvascular endothelial cells and macrophages I aim to determine any associated effects the presence of Mθ (unstimulated) and M1 (pro-inflammatory) macrophages have on PP2A and VE-cadherin. In conjunction with earlier results I hope to determine a

potential role of PP2A in pro-inflammatory induced permeability and can targeting PP2A combat the pro-inflammatory effects on PP2A and endothelial barrier function.

VE-cadherin's role in regulating endothelial barrier function is dependent on multiple factors, one of the most important being its association with accessory protein such as P120 and α- and β- catenin. The binding of which make up the VE-cadherin interactome. How PP2A is involved in regulating the VE-cadherin interactome still remains undetermined. I aim to gain further understanding into the mechanism of PP2A modulation on VE-cadherin abundance and components of the VE-cadherin interactome.

Overall, the aim of this thesis is to gain an understanding into the associated effect of PP2A modulation on VE-cadherin and brain microvascular permeability. While also determining if this association is involved in pro-inflammatory induced permeability. Allowing for an in-depth understanding into the maintenance of brain microvascular integrity and unveil potential therapeutic targets to prevent breakdown of this barrier.

Chapter 2

2 Materials and Methods

2.1 Consumables

Prouct	Manufacturer
6-well Plate	Sarstedt
12-well plate	Sarstedt
24-well plate	Sarstedt
96-well plate	Sarstedt
Cell scrapper	Sarstedt
Chromatography paper	Sarstedt
Cryogenic Vials, 1.5mL	Corning
PCR 8 tube strip and caps (200 µL)	Applied Biosciences
Polyinylidene difluoride (PVDF) membrane	GE Healthcare
Serological pipettes	Sarstedt
24-well transwell inserts	Merck Millipore
T75-cell Culture flasks (filtered cap)	Starstedt
Filtered pipette tips (2, 10, 20, 200, 1000 µL)	Fischer Brand
Pipette tips (2, 10, 20, 200, 1000 µL)	Startedt

Table 2.1 List of consumables

2.2 Equipment

Equipment	Company, Model
MX3000p, Real Time PCR machine	Applied Biosystem
Analytical balance	Mettler, AE240
Autoclave	Dixon
Fluorescence microscope	EVOS fl
Automated pipettes	Gilson, Inc. (2 µl, 10µl, 100µl, 200µl, 1000 µl, 5000 µl, Pipetman Ultra 8-channel (20-300 µl))
Centrifuge	Hettich Zentrfugen, EBA 12R/mikro 22R
Digital imaging	Fusion Fx imaging system, Vilber Lourmat
Freezer (-80°C)	Thermofischer Scientific , Revco Valure Plus
Gel electrophoresis system	Bio-Rad, Mini-Protean
Incubator (37°C, 5% CO2, 95% rh)	HERAcell 240i
Laminar flow hood	Mason Technology, BioBan 48
Luminometer	Thermofischer Scientific, Fluoroskan AscentFL
Microplate reader	BioTek EL 808
Microplate washer	BioTek ELx405
Neubauer haemocytometer, improved	BRAND GMBH + CO KG, Blaubrand ®
pH meter	Mettler-Toledo Inc., MP320
Thermocycler	MJ Research Inc, PTC-100
Heat Block	ThermoScientific

Table 2.2 List of equipment

2.3 Cell lines

2.3.1 hBMECs

hBMECs are primary human brain microvascular endothelial cells isolated from human brain cortex tissue and were purchased from Cell Systems Inc. USA. These are adherent cells grown in endothelial specific growth medium with 5% BSA. Cells were received at passage 4 and were not used passed 10 population doublings.

2.3.2 hCMEC/D3

hCMEC/D3 are an immortal cell line derived from the human temperal lobe microvessels. The primary cells isolated were immortalised by lentiviral vector transduction with the catalytic subunit of human telomerase and SV40 large T antigen, followed by extensive characteration for brain endothelial phenotype. These are adherent cells also grown in endothelial cell specific medium with 5% BSA. Cells were obtained from Dr. M. Campbell.

Chapter 3

3 VE-Cadherin Regulation by Okadaic acid, CIP2A and SET in Human Brain Microvascular Endothelial Cells

3.1 Introduction

The microvascular endothelium is a protective barrier between the systemic circulatory system and the neuronal network of the brain. Diminution of its integrity manifests as an increase in permeability and contributes to the progression of neurological conditions such as Alzheimer's disease, stroke, Parkinson's disease and bacterial meningitis [54, 612]. Fidelity of the endothelium is largely maintained through tight junctions (TJs: claudins, occludin and JAM) and adherens junctions (AJ: cadherins) [613].

In vertebrates there are approximately 20 classical cadherins, subdivided into type I and type II cadherins [614] each of which is expressed in a distinct tissue specific pattern [615]. Endothelial cells express a cell-type-specific cadherin called VE-cadherin and neuronal cadherin (N-cadherin), however the latter does not localise at cell-cell contacts [214]. In endothelial cells, VE-cadherin mediates cell-cell adhesion, maintains barrier function, facilitates angiogenesis and is directly and indirectly involved in modulating intracellular signalling associated with cell dynamics and cell cycle progression [616]. The cytoplasmic tail of VE-cadherin interacts with several proteins including p120-catenin, β-catenin, and plakoglobin. p120-catenin and β-catenin shuttle between the membrane and nucleus to regulate gene expression. More broadly, the VE-cadherin interactome also encompasses growth factor receptors (FGFR, VEGF2), signalling molecules (PKCζ, PIK3) and protein phosphatases (PP2A, PTP1B) [617, 618].

To date much of the data investigating the role of protein phosphatases in regulating VE-cadherin have focused on tyrosine phosphatases [619] rather than serine/threonine phosphatases. However, Kasa *et al*. report that inhibition of PP2A by okadaic acid or fostriecin caused β-catenin, a VE-cadherin accessory protein, to translocate from the membrane to the cytoplasm concomitant with phosphorylation at Ser^{552} [620]. Importantly, VE-cadherin and β-catenin, associate with the Bα regulatory subunit of PP2A in a pull-down assay [620].

PP2A is a ubiquitously expressed protein serine/threonine phosphatase (PSP) that functions as a trimeric protein complex consisting of a catalytic subunit (C), a scaffold subunit (A), and one of the several regulatory subunits (B). In mammals, the catalytic and scaffolding subunits exist as α and β isoforms [441] while there are four B subunit families, each with several isoforms or splice variants. The diversity of these subunits allows for the large number of substrate specificities [445]. The catalytic subunit contains a conserved amino acid sequence (TPDYFL), which is a target for post-translational modification. Receptor tyrosine kinases are responsible for the phosphorylation of Y^{307}, preventing the assembly of the holoenzyme and inhibits its activity [515]. This region can also be modified by the addition of a methyl group at Leu^{309} by leucine methyl transferase 1 (LCMT-1, which increases the activity of PP2A, and is removed by phosphatase methylesterase 1 (PME-1) [621-623]. PP2A activity is also modulated through the action of 2 endogenous inhibitors, CIP2A and SET. CIP2A inhibits PP2A by interacting with the scaffolding and regulatory subunits, while SET phosphorylates Thr^{307} of PP2Ac inhibiting catalytic activity [624].

As there is a paucity of data pertaining to the regulation of endothelial cell adherence through the presence of VE-cadherin by PP2A, the aims of the present study were to: 1) investigate if VE-cadherin is modulated by okadaic acid and CIP2A/SET, and if so determine the underlying mechanism, and 2) establish if this alters permeability of the blood brain barrier in a model system.

3.2 Materials and Methods

3.2.1 Materials

Okadaic acid was purchased from Calbiochem (Carrigtwohill, Ireland) and FTY-720 was obtained from Cayman Chemical (Hamburg, Germany). MG132 was purchased from VWR (Dublin, Ireland). Anti-VE-Cadherin (C-19), LCMT-1 (C-8), PME-1 (A-10), demethylated-PP2A-C (4B7), phosphorylated-PP2A-C (F-8) and HRP-conjugated β-actin antibodies were acquired from Santa Cruz Biotechnology (Dublin, Ireland). Goat anti-mouse and swine anti-rabbit HRP-conjugated secondary antibodies were purchased from DAKO, Agilent Technologies (Cork, Ireland). Anti-PP2A (c subunit, clone 1D6) was procured from Merck Millipore (Carrigtwohill, Ireland). DNA primers were obtained from IDT (Integrated DNA Technologies, Belgium). All other chemicals were obtained from Sigma Aldrich (Ireland) unless otherwise specified. The SET expression plasmid pcDNA-SET-FLAG-HA was a gift from Prof Judy Lieberman, Harvard Medical School, USA, while the pcDNA3.1_CIP2aflag_WT plasmid (pCIP2A) a gift from Prof J Westermarck, University of Turku, Finland. The pcDNA.3.1 (pDNA.3.1) empty vector was obtained from Dr. Steven Grey, Trinity College, Dublin.

3.2.2 Cell Culture

Human brain microvascular endothelial cells (hBMEC; Cell systems, WA, USA) and hCMEC/D3 (obtained from Dr Mathew Campbell) were cultured in EndoGRO-MV culture medium containing 5 % foetal bovine serum (FBS), supplemented with 100 U/mL penicillin, 100 mg/mL streptomycin and ciprofloxacin (10 µg/mL) respectively. All cell cultures were maintained at 37 ^{o}C in a humidified atmosphere containing 5% CO_2, and all experiments were performed under serum free conditions.

3.2.3 Cell Viability

HBMECs were seeded in 96-well plates and exposed to okadaic acid (OA, 10 nM), FTY-720 (5 µM), dimethylsulphoxide (DMSO; 0.01% v/v, solvent control), doxorubicin (DOX; 5 µM, positive control) or medium for 24 h. After 22 h, MTT [3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (5 mg/mL) was added, and the cells incubated for a further 2 h. At the end of the incubation period, the

culture medium was removed, and the purple formazan deposits dissolved in DMSO. Formazan production was quantified by spectrophotometry at a wavelength of 540 nm with a reference wavelength of 650 nm (BioTek, EL 808, Bedfordshire, UK).

3.2.4 Semi-Quantitative Real-Time PCR

Cells were grown in 6-well plates (2 x 10^5 cells per well) and total RNA isolated using TRI Reagent™ (Sigma Aldrich). Following treatment with DNase I (Sigma Aldrich), RNA was reverse transcribed using random hexamers and RevertAid reverse transcriptase (Thermofisher, Dublin, Ireland). mRNA expression was analysed by semi-quantitative PCR using target specific primers (Table 3.1) and Sybr green GoTaq DNA polymerase (Promega) on a Mx3000P qPCR system (Agilent Technology). Gene expression was quantified using the comparative Ct method [$2^{-\Delta\Delta Ct}$]. For each primer set, a no template control and a no RT control were included. Validity of the primer sequences was verified by nucleotide search (Primer-BLAST; NCBI), while the specificity and size of the amplicons were checked using a dissociation curve and gel electrophoresis followed by UV trans-illumination (Fusion Fx imaging system, Vilber Lourmat, Marne-la- Vallée Cedex, France).