DEVELOPMENT OF A LIVE-CELL ASSAY PLATFORM TO STUDY PROTEIN-PROTEIN INTERACTIONS OF MICROTUBULE-ASSOCIATED PROTEIN TAU

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Abnormal phosphorylation and aggregation of the microtubule-associated protein Tau are neuropathological hallmarks of various neurodegenerative disease called tauopathies, such as Alzheimer's disease and frontotemporal dementia. Numerous complex cellular mechanisms are involved in regulation of Tau phosphorylation but remain incompletely understood at the molecular level. The development of a novel live-cell reporter system based on protein-fragment complementation assay (PCA) enables studying dynamic changes in the status of Tau phosphorylation. In this assay, complementary fragments of *Gaussia princeps* luciferase protein are fused with Tau and Pin1 (peptidyl-prolyl *cis-trans* isomerase) proteins, and serve as a reversible real-time sensor of protein-protein interaction. Pin1 has an essential role regulating the dephosphorylation of Tau at multiple disease-associated, proline-directed phophorylation sites in neurons. The PCA assay system suits well for studying dynamic protein-protein interactions of Tau in live cells. In a chemical library screen performed using this assay, several γ-aminobutyric acid type A ($GABA_A$) receptor modulators were identified as novel regulators of Tau phosphorylation regulators. These structurally distinct $GABA_A$ receptor modulators increased the Tau-Pin1 interaction and promoted specific phosphorylation of Tau at the AT8 epitope (Ser199/Ser202/Thr205) in mature cultures of cortical neurons. The increase of this specific Tau phosphorylation induced by $GABA_A$ active molecules was associated with reduced binding to protein phosphatase 2A, a major phosphatase contributing to Tau dephosphorylation, without any reduction of enzymatic activity of protein phosphatase 2A *per se* in a cell-free assay. These data provide new insight in the different mechanisms of Tau regulation and functions, which is essential for more detailed understanding of basic neurobiology and mechanisms of neurodegeneration.

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Mikrotubulus-assosioituneen Tau proteiinin epänormaali fosforylaatio ja aggregoituminen ovat ominaisia neuropatologisia tunnusmerkkejä useille hermorappeumasairauksille, tauopatioille, kuten Alzheimerin tauti ja frontotemporaalinen dementia. Useat monimutkaiset molekulaariset mekanismit ja post-translationaaliset modifikaatiot jotka vaikuttavat Tau:n fosforylaatioon ovat tällä hetkellä vaillinaisesti ymmärrettyjä. Uuden solupohjaisen reportterisysteemin kehitys, joka pohjautuu proteiinifragmenttien komplementaatiomenetelmään, mahdollistaa Tau:n fosforylaation dynaamisten muutosten tutkimisen elävissä soluissa. Kehitetyssä menetelmässä *Gaussia princeps*-lusiferaasiproteiinin komplementaariset osat on yhdistetty Tau ja Pin1 (peptidyyli-prolyyli *cis/trans* isomeraasi) proteiineihin, ja reportteriproteiinin aktiivisuus toimii sensorina proteiini-proteiinivuorovaikutuksen muutoksissa. Hermosoluissa Pin1 on keskeinen Tau:n defosforylaation säätelijä useissa tautispesifisissä proliini-ohjatuissa fosforylaatio tähteissä. Kehitetty menetelmä soveltuu hyvin Tau:n proteiini-proteiinivuorovaikutusten tutkimiseen. Tätä menetelmää käyttäen suoritetulla kemiallisten aineiden seulonnalla löydettiin useita γ-aminovoihappo tyyppi A:n ($GABA_A$) reseptorimodulaattoreita, jotka säätelevät Tau:n fosforylaatiota. Seulonnassa idenfifioidut rakenteellisesti toisistaan poikkeavat $GABA_A$–reseptorimodulaattorit lisäsivät Tau-Pin1 vuorovaikutusta, sekä lisäksi indusoivat Tau:n fosforylaatiota erityisesti AT8 fosfoepitoopissa (Ser199/Ser202/Thr205) kypsissä kortikaalisten hermosolujen viljelmissä. Näiden $GABA_A$–aktiivisten molekyylien indusoima Tau:n fosforylaatio liittyi vähentyneeseen sitoutumiseen proteiinifosfataasi 2A:n (PP2A) kanssa, joka on Tau:n defosforylaation kannalta keskeinen fosfataasi. PP2A:n entsyymiaktiivisuuden vähenemistä ei kuitenkaan havaittu soluvapaassa aktiivisuusmäärityksessä. Erilaisten Tau:n fosforylaatioon ja biologisiin tehtäviin vaikuttavien mekanismien tutkiminen on välttämätöntä yksityiskohtasemman ymmärryksen saavuttamiseksi niin perus neurobiologiassa, kuin myös hermorappeumaan johtavissa mekanismeissa.

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1 INTRODUCTION

In the complex cellular environment, an immense amount of molecular interactions defines the fate of a cell. Particularly, dynamic network of protein-protein interactions (PPIs) govern to the functionality of the cell mostly via the formation of multi-protein complexes. Regardless of intense research in this area, the interaction patterns of proteins within the cell are poorly understood. However, aberrations in PPIs are widely recognized to associate with numerous pathological disorders which affords an opportunity for developing novel therapeutic strategies by modulating the pathophysioligically relevant PPIs (Arkin & Wells 2004).

Cerebral aggregation of misfolded proteins is a common pathological feature of several neurodegenerative disorders (e.g. Lee et al. 2001; Ballatore et al. 2007). A group of neurodegenerative disorders that are charactherized by intracellular inclusions of abnormally phosphorylated microtubule-associated protein tau (MAPT) are collectively known as tauopathies. These prominent and progressive intracellular tau inclusions, called neurofibrillary tangles (NFTs), are accumulations of filamentous tau deposits. Lesions in the central nervous system (CNS), including neuronal degeneration and consequent brain dysfunction, are neuropathological hallmark features of these dementias and movement disorders, such as Alzheimer's disease (AD) and frontotemporal dementias, that are linked to aberrant tau phosphorylation. Despite of the incomplete knowledge of these specific lesions mentioned, the exact temporal stages of neuropathology and even the divergence of disease phenotypes of various tauopathies, the synergistic contribution of aberrant tau functions to tau-mediated neurodegeneration has become more evident. Importantly, the combination of both functional abnormalities, toxic gain-of-functions and loss of normal tau function(s) is capable and sufficient to cause neurodegenerative disorders independently of other disease-specific aberrations (e.g. Spillantini et al. 1998; Hutton et al. 1998).

Establishing the specific roles of various protein aggregates to the onset and progression of the disease is essential for discovering the mechanism(s) of disease pathology (Ballatore et al. 2007). Howerer, investigation of these early molecular events contributing to the disease onset is highly challenging. There are many aspects that hinder the studies of these dynamic processes that may take even years to manifest. In the case of tau, there are some contradictions of cause-and-effect functions of diverse neurotoxic tau species and their contribution to disease pathology and the possible pre-aggregated or pre-fibrillary forms further complicates the study designs and may cause misinterpretation of data. Also, in addition to the well-established function of tau in promoting the assembly and stabilizing the stucture of microtu-

bules, tau may also possess other functions that are not that well characterized, for example interactions with other proteins and enzymes (Buee et al. 2000). Furthermore, besides abnormal phosphorylation tau undergoes numerous other post-translational modifications of which some could have more or less pronounced effects on the formation of NFTs (Gong et al. 2005). Thus, combined effects of these modifications comprise a complicated system to alter the tau protein, and this system likely contributes to attenuation or exacerbation of the disease onset and progression of tau-mediated neurodegeneration.

Identification of multiple events in the cascade finally leading to tau-driven neurodegeneration is instrumental in order to search for novel therapeutic approaches and drug candidates for tauopathies (e.g. Ballatore et al. 2007). As PPIs are vital for cellular function, investigating the protein-protein interactions of tau may eventually improve our understanding of this highly complex pathway and shed new light towards the key factors to arrest, or at least to slow down the progession of these devastating neurodegenerative disorders. Because of many of these disases have a great tendency of being hardly detected in their early pathogenesis, it is high of an importance to try to address the investigation methods towards these early events.

2 BIOLOGY OF TAU PROTEIN

2.1 Structure and functions

Microtubules (MTs) are components of the cytoskeleton, a cellular structure that is vital in axon and dendrite formation, which both contribute to the intracellular transport of numerous molecules and to neurotransmission (e.g. Meraz-Rios et al. 2010). Microtubule-associated protein tau (MAPT) is a major cytoskeletal-associated protein, which is encoded by a single gene that is located on human chromosome 17q21 containing 16 exons (figure 1) (Neve et al. 1986). The primary transcript of tau contains 13 exons, since exons 4A, 6 and 8 are specific for peripheral tau and are not present in the human brain mRNA (Buee et al. 2000). Exons 1, 4, 5, 7, 9, 11, 12 and 13 are constitutive exons of tau protein, unlike exons -1 and 14, which are both transcribed but not translated into a protein (Goedert et al. 1989a).

There are six tau isoforms found in the human brain, produced by alternative splicing of exons 2, 3 and 10 in tau mRNA (figure 1) (Goedert et al. 1989a; Andreadis et al. 1992), from which exon 3 is exon 2 dependent, i.e. exon 2 can appear independently but exon 3 never appears if exon 2 is absent (Andreadis et al. 1995). These six tau isoforms (with exon combinations of 2-3-10-; 2+3-10-; 2+3+10-; 2-3-10+; 2+3-10+; 2+3+10+) vary in lenght ranging from

352 to 441 amino acids (Goedert et al. 1989a; Goedert et al. 1989b; Kosik et al. 1989). Tau protein can be structurally divided into two domains: the projection domain and the microtubule-binding domain (MBD) (e.g. Wang & Liu 2008; Meraz-Rios et al. 2010). The projection domain, which is located at the amino-terminal end of tau molecule, is suggested to project from the microtubule surface (Hirokawa et al. 1988), and can be further divided into two regions (e.g. Wang & Liu 2008; Meraz-Rios et al. 2010). The C-terminal region of the projection domain is enriched with proline residues and is positively charged, whereas the N-terminal part has a positive charge and contains mainly acidic residues. In the N-terminal region there are two or one or zero 29 amino acid sequences (2N, 1N, 0N, respectively) encoded by exons 2 and 3. This domain is followed by proline-rich region that modifies the lenght of the N-terminus of the projection domain, thereby naturally modifying the lenght of the entire tau protein (e.g. Buee et al. 2000). Although the exact function of the projection domain is yet to be determined, it has been reported to interact with cytoskeletal proteins other than tau, e.g. neurofilaments and plasma membrane (Hirokawa et al. 1988; Brandt et al. 1995). Also, a regulatory role has been described for proline-rich region in the interaction between tau and microtubules via phosphorylation (e.g. Lee et al. 2004).

Among the neuronal microtubule-associated protein (MAP) family including MAP1, MAP2 and tau (e.g. Iqbal et al. 2005), tau is functionally the major microtule-associated protein in neurons (Weingarten et al. 1975). The main function of tau is to stabilize and promote the assembly and disassembly of microtubules. The binding of tau to microtubules occurs via microtubule-binding repeats encoded by exons 9-12 that are localized in the MBD at the C-terminal part of tau protein (Lee et al. 1989). Also, MBD can be further divided into microtubule-binding domain *per se* and to an acidic region that forms the C-terminal region of MBD (e.g. Wang & Liu 2008; Meraz-Rios et al. 2010). The actual MBD contains either three (3R-tau, repeats R1, R3, R4) or four (4R-tau, repeats R1, R2, R3, R4) repetitive sequences of 31 or 32 amino acid residues, which are similar but not identical (Lee et al. 1989; Iqbal et al. 2009). These different isoforms, i.e. whether the R2-repeat is absent (3R-tau) or present (4R-tau), are result of alternative splicing of exon 10 that encodes the R2-repeat (e.g. Andreadis et al. 1992; Hernandez & Avila 2007).

All the six isoforms of tau (2N/4R, 1N/4R, 0N/4R, 2N/3R, 1N/3R, 0N/3R) have been found in the adult human brain, but only the shortest isoform, 0N/3R, is expressed in fetal human brain (Kosik et al. 1989; Goedert et al. 1989b). The inclusion of additional R2-repeat in the 4R-tau and the presence of one or both N-terminal inserts (1N and 2N) enhances the microtubule-binding affinity, thereby making the longest isoform (2N/4R) the most efficient

in promoting MT-assembly (e.g. Lu & Kosik 2001; Alonso et al. 2001b). Additionally to R2-repeat, a specific peptide sequence within the inter-region between repeats R1-R2 is suggested to be the most potential region for inducing microtubule polymerization (Goode & Feinstein 1994).

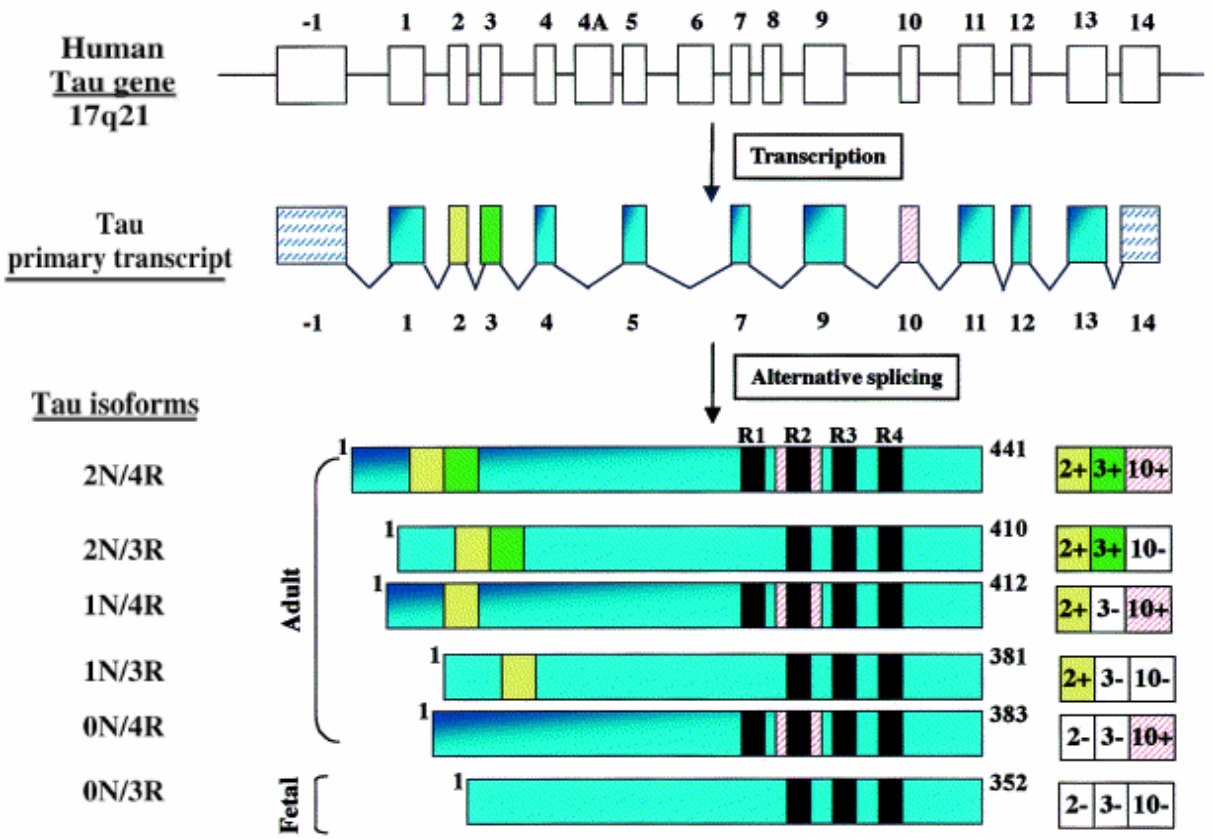


Figure 1. Tau encoding *MAPT* gene, primary transcript and domain structures of six human tau isoforms expressed in the central nervous system. The human *MAPT* gene that encodes tau protein is located at the chromosome 17 position 17q21. From the 16 exons of the *MAPT* gene, 13 exons are transcribed in the primary transcript, since exons 4A, 6 and 8 are specific for peripheral tau and are not present in the human brain mRNA. Exon -1, which is part of the promoter, and exon 14 are both transcribed but not translated into a protein. There are six tau isoforms found in the human brain produced by constitutive exons 1, 4, 5, 7, 9, 11, 12 and 13, and alternative splicing of exons 2, 3 and 10. Differences between the six human tau isoforms are due to presence or absence of one or two amino-terminal inserts of 29 amino acids (0N, 1N, 2N) encoded by exons 2 and 3 combined with carboxy-terminal inserts of either 3 (R1, R3 and R4) or 4 (R1-R4) repeat-regions. R2 repeat-region is encoded by exon 10. These six tau isoforms vary in lenght including 352, 381, 383, 410, 412 or 441 amino acids (with exon combinations of 2-3-10-; 2+3-10-; 2-3-10+; 2+3+10-; 2+3-10+; 2+3+10+, respectively). All tau isoforms have been found in the adult human brain, but only the shortest isoform 2-3-10- (0N/3R), is expressed in fetal human brain (modified from Bueé et al. 2000).

Besides the regulatory role of tau in microtubule dynamics, other possible physiological functions have been reported. Tau has been found to be localized in the cell nucleus in the

human brain, and in contrast to cytosolic tau, nuclear tau is found to be less soluble, implicating the role of specific modifications which may either post-translational or mediated via interaction with other proteins (Brady et al. 1995). Moreover, tau is reported to bind RNA through MBDs leading to a subsequent induction of paired helical filament (PHF) formation (Kampers et al. 1996). Tau is also found to contribute to cell viability by antagonizing apoptosis (Li et al. 2007) and to interfere with binding of kinesin and kinesin-like motor proteins to microtubules and thereby disrupting the axonal transport (Tatebayashi et al. 2004). Until to date and according to its physiological functions, tau has been mainly considered as an axonal protein. However, a recent study has reported a novel dendritic role of tau (Ittner et al. 2010). These results suggest that tau mediates Aβ-toxicity in AD. The postsynaptic targeting of Fyn, a Src-family tyrosine kinase, that involves the interaction of Fyn with the projection domain of tau, was found to be reduced in knockout mice resulting in decreased phosphorylation of its substrate NMDA receptor. This reduced phosphorylation of NMDA receptors decreases NMDA receptor-mediated excitotoxicity thereby mitigating the Aβ-toxicity.

2.2 Regulation of phosphorylation and dephosphorylation

The biological activity of tau, like other phosphoproteins, is regulated by the degree of its phosphorylation (e.g. Kopke et al. 1993; Alonso et al. 1994). Like tau expression, also phosphorylation of tau is developmentally regulated (Goedert et al. 1993). The shortest isoform (0N/3R) of tau is highly phoshoporylated in the fetal and postnatal human brain, whereas its phosphorylation is markedly decreased within the healthy adult brain. Interestingly, tau is also functionally regulated, for example as seen in increased phosphorylation states during the mitotic stage of cell cycle (Delobel et al. 2002). Hence, both developmental and functional regulation of tau phosphorylation may play a crucial role in the regulation of microtubule stabilization and dynamics during the normal neurite formation and other MT-dependent functions (e.g. Wang & Liu 2008). Furthermore, tau proteins that are hypophosphorylated or hyperphosphorylated loses their ability to bind to microtubules, thus indicating that phosphorylation at certain sites at tau is required for optimal binding to microtubules and maintain the function to stabizile their structure (Garcia de Ancos et al. 1993). In contrast, phosphorylation at specific sites may prevent this stabilizing role of tau and may promote its self-aggregation.

Tau phosphorylation is catalyzed by several protein kinases which each has specific preference towards specific phosphorylation regions or sites of tau (table 1) (e.g. Liu et al. 2007). There are more than ten kinases capable to phosphorylate the serine (Ser) and threonine (Thr)

residues of tau *in vitro*. The kinases that phosphorylate tau can be further divided into two distinct groups: proline-directed protein kinases and non-proline-directed protein kinases (PDPKs and NPDPKs, respectively), according to their motif-specifity (e.g. Wang & Liu 2008; Meraz-Rios et al. 2010). The PDPKs includes kinases such as GSK-3β, CDK5, DYRK1A, MAPK and ERK1/2, of which the GSK-3β is the most strongly associated to contribute to the abnormal hyperphosphorylation of tau in AD pathogenesis (e.g. Grimes & Jope 2001; Gong et al. 2005; Wang & Liu 2008). The NPDPKs involved in tau phoshorylation include e.g. PKA, PKC and CaMKII. Opposite to phosphorylation, all the major protein phosphatases (PPs) excluding PP2C, can dephosphorylate tau (Liu et al. 2005). Among the PPs (PP1, PP2A, PP2B, PP5), PP2A contributes with the highest efficacy on tau dephosphorylation. In addition to kinases and phosphatases mediating the tau phosphorylation, its conformational state regulates the rate and extent of its phosphorylation (e.g. Alonso Adel et al. 2004). The conformational changes may promote tau as a more suitable substrate for kinases and or may decrease the efficacy of dephosphorylation by making tau worse substrate to protein phosphatases (e.g. Iqbal et al. 2005). Priming phosphorylation of tau by certain NPDPKs markedly stimulates the subsequent phosphorylation by PDPKs thereby promoting hyperphosphorylation (e.g. Liu, S.J. et al. 2004).

The longest human brain tau isoform (2N/4R) consisting of 441 amino acids cointains 80 putative Ser and Thr residues altogether, from which more than 30 has been identified to be phosphorylated in PHF-tau of AD brain (e.g. Gong et al. 2005; Wang & Liu 2008). The majority of these sites are localized in the proline-rich region (residues 172-251, numbering according to the longest isoform 2N/4R) and C-terminal region (residues 368-441) flanking the MBD, with the exception of sites Ser262 (R1), Ser285 (R1-R2 inter-repeat), Ser305 (R3-R4 inter-repeat), Ser324 (R3), Ser352 (R4) and Ser356 (R4) which are localized in the MBD (e.g. Gong et al. 2005; Liu et al. 2007; Wang & Liu 2008). The hyperphosphorylation of MBD-localized sites has been proposed to have more detrimental effect on microtubule assembly promotion and stabilization compared to the other sites of tau.

The hyperphosphorylation of tau could be the result of impaired balance between the phosphorylation and dephosphorylation caused by upregulation of kinase activities and/or downregulation of phosphatase enzyme activities (e.g. Gong et al. 2005). However, among the over 30 sites of PHF-tau that has been found to be phosphorylated, the crucial sites involved in the development of AD pathology has been poorly understood. Also, there has been a debate for years whether the abnormal hyperphosphorylation *per se* is sufficient to induce neurotoxicity and neurofibrillary pathology seen in AD and related tauopathies. Interestingly,

the results of recent study suggests that combined phosphorylation of tau at sites Thr212, Thr231 and Ser262 can induce tau aggregation, cause disruption of MT-network and neurotoxicity and concomitant neurodegeneration and apoptosis (Alonso et al. 2010). These results provides *in vitro* evidence of the contribution of abnormal tau hyperphosphorylation to neurofibrillary degeneration and identifies some phosphorylation sites that induce the conversion of tau to toxic molecule thereby losing its ability to maintain normal function. Furthermore, additional sites that are suggested to facilitate the conversion of tau into toxic protein includes Ser199, Ser202, Thr205, Ser235, Ser356, Ser396, Ser404 and Ser422 (Alonso Adel et al. 2004).

2.2.1 Kinases

2.2.1.1 GSK-3β

Glycogen synthase kinase-3 (GSK-3) is a proline-directed (serine or threonine preceding proline) serine/threonine protein kinase, which in mammals, is encoded by two genes, *gsk-3α* and *gsk-3β* (Woodgett 1990). The two isoforms of the end products of these two genes, GSK-3α (~51 kDa) and GSK-3β (~47 kDa), are abundant in the brain. These two GSK-3 isoforms are highly homologous, but have differencies in their N- and C- terminal regions (e.g. Avila et al. 2010). As a crucial regulatory enzyme, GSK-3 has multiple functions including phosphorylation of several substrates and regulating various physiological processes such as signal transduction and glycogen metabolism (e.g. Grimes & Jope 2001).

GSK-3 activity is modified by its phosphorylation of an N-terminal serine (Ser21 in GSK-3α and Ser9 in GSK-3β) (Jope & Johnson 2004). Protein kinase A (PKA) and Akt are few of the group of priming kinases which can phosphorylate these serines and, thereby, reduce the activity of GSK-3. In contrast to reducing the activity of GSK-3, tyrosine phosphorylation (Tyr279 in GSK-3α and Tyr216 in GSK-3β) increases the enzyme activity. Furthermore, many of the GSK-3 substrates must be primed (pre-phosphorylated) for subsequent phosphorylation by GSK-3, hence the phosphorylation state of the substrates also regulate GSK-3 activity towards them. Other way to regulate GSK-3 activity includes its interaction with other proteins and protein complexes (Avila et al. 2010) and also its subcellular localization, since GSK-3 has been found in various cellular compartments (Grimes & Jope 2001).

Besides that GSK-3 interacts with APP, another hallmark protein in AD, it has been reported that, especially GSK-3β isoform, can phosphorylate tau on several sites (e.g. Grimes & Jope 2001). As a one of the main kinases phosphorylating tau, GSK-3 participates to regula-

tion of phosphorylation equilibrium of tau under normal physiological conditions. In contrast, it highly contributes to abnormal phosphorylation of tau and subsequent dissociation of tau from the microtubulus. However, the phosphorylation of primed sites of tau by GSK-3β seems to have more significant effect on its interaction with microtubulus compared to phosphorylation at non-primed sites (Cho & Johnson 2003). There have also been implications that Ser9-phosphorylated GSK-3β, which is associated with reduced kinase activity, can phosphorylate tau through unknown mechanism (Yuan et al. 2004).

2.2.1.2 CDK5

Cyclin-dependent kinase 5 (CDK5) has a crucial role in the development and maintenance of the central nervous system by phosphorylation of a large number of substrates (Cruz & Tsai 2004a). In addition to GSK-3, CDK5 is another main proline-directed serine/threonine kinase regulating the phosphorylation state of tau. For enzyme activity, CDK5 has to associate with its regulatory subunits, p39 and p35, for its normal functions. The activators p35 and p39 are mainly expressed in post-mitotic neurons thereby restricting the CDK5 activity mostly to the central nervous system (Lew et al. 1994).

The neurotoxicity of CDK5 deregulation contributes to pathogenesis of various neurodegenerative disorders, such as AD (Cruz & Tsai 2004b). Under neurotoxic conditions (e.g. ischemic or oxidative damage) calpain cleaved truncated forms of the activators p39 and p35, p29 and p25, respectively, are generated (Kusakawa et al. 2000; Patzke & Tsai 2002). These cleaved forms are more stable thereby prolonging the activity of p25/p29-CDK5 heterodimer. Also, by binding to p25, subcellular localization of p25-CDK5 complex is changed compared to p35-CDK5 complex (Cruz & Tsai 2004b) and the substrate specifity is altered (e.g. Patrick et al. 1999). The generation and accumulation of p25 e.g. in AD brains causes tau hyperphosphorylation and induces cytosceletal disruption and apoptosis in neuronal cells (Patrick et al. 1999).

2.2.1.3 DYRK1A

The dual-specifity tyrosine (Y)-phosphorylation-regulated kinase 1A (DYRK1A) is a proline-directed serine/threonine protein kinase that has recently been related to tau phosphorylation (e.g. Ryoo et al. 2007). DYRK1A is a multifaceted enzyme that phosphorylates several proteins and has dual substrate specifity: it autophosphorylates on the threonine231 residue in the activation loop of the catalytic domain for its self-activation (Himpel et al. 2001) and the tar-

geted phosphorylation of serine/threonine residues of various proteins (Himpel et al. 2000). It has been also suggested, that DYRK1A primes various substrates, e.g. tau, to be subsequently phosphorylated by GSK-3 (Woods et al. 2001).

The gene encoding the kinase is located in the human chromosome 21, the same chromosome which full or partial trisomy causes Down syndrome (DS) (e.g. Wiseman et al. 2009). Many of the genes that are encoded in this chromosome are, due to an additional copy, overexpressed leading to various deficits in development including mental retardation and impaired learning and memory, wherein DYRK1A is suggested to play a critical role (Ahn et al. 2006). Almost all the patients with DS develop AD-like dementia and brain lesions similar to AD pathology by the age of 40, decades earlier than generally shown among population (e.g. Park et al. 2009). Besides tau, DYRK1A also phosphorylates amyloid precursor protein on threonine 668 and presenilin-1, which in turn could elevate the level of amyloid-β production and thereby senile plaque formation (Ryoo et al. 2008; Ryu et al. 2010). Considering these aspects, overexpression of DYRK1A has been suggested to have a crucial role in development of AD pathology in DS and implicated as a functional link between AD and DS (Ryoo et al. 2007; Ryu et al. 2010).

2.2.1.4 Fyn

Unlike the kinases discussed so far, Fyn is a tyrosine kinase (Resh 1998). Fyn is a member of Src tyrosine kinase family with diverse biological functions, such as brain function regulation and cell adhesion signalling. Tyrosine phosphorylation of tau by Fyn has been reported to occur after amyloid-β exposure (Williamson et al. 2002) and during the development of neurodegeneration, although it might not largely affect the microtubule-binding properties of tau (Lee et al. 2004). Regardless of its slight contribution to microtubule-binding efficacy of tau, tyrosine phosphorylation has been suggested to have distinct mechanisms to participate in the regulation of tau phosphorylation. Tyrosine phosphorylation may be an indicator of relocalized tau towards cellular compartments where activated kinases are present, or, an indicator of activated src family kinases capable of activating the subsequent phosphorylation of tau by serine/threonine kinases. Regarding to the role of Fyn in neurodegeneration, it has been recently suggested that axonal protein tau may have a role in Fyn transport in dendritic postsynapse, thereby mediating the synaptotoxicity of Aβ via the N-methyl-D-aspartate (NMDA) receptor (Ittner et al. 2010).

2.2.1.5 PKA

Protein kinase A (cAMP-dependent protein kinase, PKA) is a non-proline-directed multisubstrate protein kinase, which participates in various pivotal signalling pathways (Walsh & Van Patten 1994). The elevated level of intracellular cAMP and the co-localization of the PKA holoenzyme and the specific substrates, are the key regulatory elements on inducing the activation of PKA-catalyzed signal transduction pathway. The role of PKA on tau phosphorylation, like numerous other kinases, has been widely studied *in vitro*, and also, the function of PKA on tau phosphorylation has been presented *in vivo* (Liu, S.J. et al. 2004).

PKA is a priming kinase that prephosphorylates its substrates (e.g. tau) at specific serine/threonine sites and, thereby, facilitates the subsequent phosphorylation by GSK-3 or CDK-5, or both (Liu, S.J. et al. 2004; Wang et al. 2007). Furthermore, it has been suggested that contribution of PKA alone, preceeding the sequental phosphorylation by various kinases, may generate AD-like abnormal phosphorylation state of tau in a duration-independent manner (i.e. only transitory activation with specific PKA-activator could induce tau hyperphosphorylation) (Zhang et al. 2006; Wang et al. 2007). Besides PKA, other non-proline-directed protein kinases, such as calcium- and calmodulin-dependent protein kinase II (CaMKII), protein kinase C (PKC) and casein kinases, have been indicated to play roles in the regulation of tau hyperphosphorylation (e.g. Kuret et al. 1997; Yamamoto et al. 2002; Liu et al. 2003).

2.2.1.6 MAP kinases

Mitogen-activated protein kinases (MAPKs) are a family of proline-directed serine/threonine protein kinases that have been extensively studied (e.g. Schaeffer & Weber 1999). MAPK signal trasduction cascades participate in many cellular functions and typically function in a signal cascade involving at least three consecutive kinases (e.g. MAP3K, MAP2K and MAPK) (Schaeffer & Weber 1999; Kim & Choi 2010). The subsequent activation of the kinases in the signalling pathway eventually leads to phosphorylation of numerous MAPK substrates. The activation of these signalling pathways may occur through interaction between two components of the kinase or due to a result of multiple kinase signalling complex formation regulated by specific proteins (Kim & Choi 2010).

The family of mammalian MAPK includes extracellular signal-regulated kinase (ERK), c-Jun NH_2-terminal kinase (JNK, which is also known as stress activated protein kinase) and p38 (Schaeffer & Weber 1999). All of these kinases exists in various isoforms (e.g. ERK1 to ERK8) and take part in numerous cellular programs, such as apoptosis, inflammation and

other stress responses, and cell growth and differentiation. The contribution of MAPK signalling pathways to pathogenesis of wide array of disorders, consisting cancer and many neurodegenerative disorders like AD, Parkinson's disease (PD) and amyotrophic lateral sclerosis (ALS), have been established (e.g. Kim & Choi 2010).

In AD, the MAPKs have been implicated to have multiple distinct roles in disease pathogenesis, including the regulation of β- and γ-secretases, the induction of neuronal apoptosis and phosphorylation of both AD hallmark proteins, APP and tau (e.g. Munoz & Ammit 2010; Kim & Choi 2010). JNK, ERK and p38 MAPK have each been suggested to participate in aberrant tau phosphorylation in AD (e.g. Churcher 2006). Activated (phosphorylated) p38 has been shown to co-localize with accumulated tau in cortical and hippocampal brain slices of patients with AD (Zhu et al. 2000) and also in transgenic htau mice (Kelleher et al. 2007). The finding that microglial interleukin-1 (IL-1), a cytokine known to activate p38 MAPK, activates p38 in neurons, which in turn may lead to a neurofibrillary degeneration through increased tau phosphorylation, is suggested to provide a molecular link between neuroinflammation and tau hyperphosphorylation (Sheng et al. 2001).

2.2.2 Dephosphorylation

Protein phosphatases (PP) reverse the tau phosphorylation by kinases (Liu et al. 2005). The activity of PPs is regulated via various cellular mechanisms including calcium, subcellular localization of the PPs and its substrates and phosphorylation of the PP-subunits (Tian & Wang 2002). There are five serine/threonine PPs found to be expressed in the mammalian brains: PP1, PP2A, PP2B, PP2C and PP5, from which all except PP2C is known to dephosphorylate tau protein *in vitro* (Liu et al. 2005). PP1, PP2A, PP2B and PP5 all dephosphorylate the same specific tau phosphorylation sites, but the efficacy towards different sites differs largely (table 1). In the human brain, the total tau dephosphorylation activity of protein phosphateses 1, 2A, 2B and 5 is unevenly distributed; ~11%, ~71%, ~7% and ~ 10%, respectively. These partial PP contributions, in addition to the findings of decreased activity of PP2A in human AD brains, suggest PP2A to be the major tau phosphatase. PP2A regulates the phosphorylation not only by dephosphorylating tau, but it also regulates the kinases that participate in the phosphorylation of tau (Tanimukai et al. 2005).

Two distinct endogenous inhibitors, I_1^{PP2A} and I_2^{PP2A}, regulate the intracellular activity of PP2A (Tanimukai et al. 2005). I_1^{PP2A} inhibit PP2A through its catalytic subunit (PP2Ac), unlike I_2^{PP2A}, which is first cleaved into two fragments and translocated from nucleus to cyto-

plasm, where it interacts with PP2Ac and co-localizes with aggregated tau. The level of these two heat-stable proteins have been found to be increased ~20% in the AD brains and have an effect on abnormal tau hyperphosphorylation. Memantine, which is an NMDA-receptor antagonist clinically used to treat AD, has been reported to restore the ability of PP2A to dephosphorylate tau and to inhibit abnormal tau phosphorylation and neurofibrillar degeneration (Li et al. 2004).

The activity and the expression level of PP2A are notably reduced in the AD brain, which is indicated to contribute to tau hyperphosphorylation (e.g. Vogelsberg-Ragaglia et al. 2001). Taken together the reduced levels, the suggested role as the major tau phosphatase and interaction with other AD-related proteins, PP2A is considered as a potential target for drug development for AD (e.g. Tian & Wang 2002; Liu et al. 2005). Furthermore, an increase in demethylation of PP2A disrupts the regulation of its activity and may decrease the dephosphorylation of hyperphosphorylated tau, thus augmenting the importance to further studies PP2A function in AD (Zhou et al. 2008). Recently, it has been implicated that sodium selenate is a specific PP2A activator, a compound shown to reduce tau phosphorylation without any signs of neurotoxicity (Corcoran et al. 2010; van Eersel et al. 2010). Sodium selenate has been reported to markedly enhance the PP2A activity and stabilize the PP2A-tau complex, hereby providing a promising novel candidate to targeted AD treatment.

Table 1. The specific sites of tau phosphorylated and dephosphorylated by certain kinases and phosphatases. N-tau, tau from normal adult brain; AD-tau, abnormally hyperphosphorylated tau from Alzheimer's disease brains; CaMKII, calcium- and calmodulin-dependent protein kinase II; CDK5, Cyclin-dependent kinase 5; ERK 1/2, extracellular signal-regulated kinase; GSK-3β, Glycogen synthase kinase-3β; PKA, cyclic AMP-dependent protein kinase; PP1/PP2A/PP2B/PP5, protein phosphatase 1/2A/2B/5; T, threonine; S, serine; Y, tyrosine.

Site	N-tau	AD-tau	Kinases						Phosphatases			
			CaMKII	CDK5	ERK 1/2	GSK-3β	PKA	FYN	PP1	PP2A	PP2B	PP5
T39		X										
S46		X			X	X				X	X	
T50					X	X						
S131			X									
T135			X									
T149						X						
T153		X			X	X						
T175		X				X						
T181	X	X		X	X	X				X	X	X
S184						X						
S195				X		X						
S198		X				X	X					
S199	X	X		X	X	X			X	X	X	X
S202	X	X		X	X	X			X	X	X	X

T205	X	X		X	X	X			X	X	X	X
S208		X				X	X					
S210		X				X	X					
T212	X	X	X	X	X	X			X	X	X	X
S214	X	X	X				X		X	X	X	X
T217		X		X	X	X	X		X	X	X	
T220						X	X					
T231		X		X		X			X	X	X	X
S235		X		X	X	X			X	X	X	X
S237		X				X						
S238		X										
S241		X				X						
T245						X	X					
S258						X	X					
S262		X	X			X	X		X	X	X	X
S285		X				X						
S289						X						
S305		X				X	X					
S324		X				X	X					
S352		X				X	X					
S356		X	X			X	X			X	X	
T373				X		X						
S396	X	X		X	X	X			X	X	X	X
S400		X				X						
T403		X				X						
S404	X	X		X	X	X			X	X	X	X
S409		X				X	X		X	X	X	X
S412		X				X	X					
S413		X				X	X		X			
T414						X	X					
S416		X	X			X	X					
S422		X			X					X	X	
Y18		X						X				
Y29		X						X				
Y394		X						X				

2.2.3 Hyperphosphorylation and mechanism of neurofibrillary degeneration

Abnormal hyperphosphorylation of tau is the major factor contributing to tau dysfunction and neurofibrillar degeneration in AD and other related tauopathies (e.g. Grundke-Iqbal et al. 1986; Alonso et al. 1994; Iqbal et al. 2009). In contrast to normally phosphorylated tau containing 2-3 moles of phospahate per mole of tau, the phosphorylation status of hyperphosphorylated tau is 3-4 fold higher in AD brains (Kopke et al. 1993). Numerous factors, including impairment of the phosphorylation/dephosphorylation equilibrium, alterations in brain glucose metabolism and pathways mediated by Aβ (although there are controversies about the role of Aβ), are a few to mention that may induce the abnormal hyperphosphorylation of tau (e.g. Gong & Iqbal 2008). Regardless of extensive studies, the mechanism of hyperphosphorylated tau-induced neurofibrillar degeneration is not completely understood but probably

involves both loss of normal function and gain of toxic function components (e.g. Wang & Liu 2008).

Most likely due to abnormal hyperphosphorylation, tau detaches from the microtubules and is accumulated into intraneuronal tangle formations of paired helical filaments (PHF) and or straight filaments (SF), wherein it is the predominant protein subunit, and, which subsequently forms neurofibrillary tangles (NFT) (Grundke-Iqbal et al. 1986; Iqbal et al. 1989; Alonso et al. 2001a). In addition to self-assembly of all of the six tau isoforms into inert aggregates of PHFs/NFTs (Alonso et al. 2001a), the non-fibrillized cytosolic hyperphosphorylated tau also sequesters other microtubule-associated proteins (MAP1 and MAP2) and normal tau (Kopke et al. 1993; Alonso et al. 1994; Alonso et al. 1997). This toxic gain-of-function leads to inhibition of microtubule assembly and disruption in microtubule stabilization. AD-like hyperphosphorylated tau (AD-like p-tau) binds to and sequesters different tau isoforms with affinity of 2N/4R > 1N/4R > 0N/4R and 2N/3R > 1N/3R > 0N/3R, and also that 2N/4R > 2N/3R (Alonso et al. 2001b). According to the preferential sequestration of 4R-tau isoforms by AD-like p-tau, the absence of N-terminal inserts and the additional fourth microtubulebinding repeat (R2) in fetal tau isoform (0N/3R), implicates a protective role for fetal human tau from neurofibrillary degeneration. AD-like p-tau is also suggested to decrease the function of proteasomes and the PHF-tau has been found to be polyubiquitynated, which raises the question on the importance of the degradation and clearence of AD-like p-tau (Cripps et al. 2006). Furthermore, hyperphosphorylated tau compromises axonal transport by interfering with the kinesin-like motor proteins and by destabilizing microtubules, which serve as tracks in axonal transport of various organelles and proteins (e.g. Tatebayashi et al. 2004).

The role of protein kinases and different combinations of these kinases that can induce the abnormal phosphorylation of tau, have been established (Wang et al. 2007). By phosphorylating tau with various kinases, including PKA, CaMKII, GSK-3β and CDK-5, using diverse subsequental kinase treatments, normal tau was shown to hyperphosphorylate, and self-assembly into PHFs was induced. The ability of PP2A to dephosphorylate all the crucial sites needed for AD-like p-tau self assembly, was also reported, thereby augmenting its role as the major tau dephosphorylating protein phosphatase. Additionally, the rephosphorylation of PP2A-dephosphorylated AD-like p-tau by different kinase combinations restored the properties to form PHFs/NFTs, implicating that abnormal tau hyperphosphorylation is reversible event and requires more than one protein kinase.

The sequential pattern of various AD-associated phosphorylation sites has been studied *in vitro* (Bertrand et al. 2010). Besides the priming effects of different kinases, the sequential phosphorylation of specific tau sites could also regulate the overall phosphorylation status of tau via various cascades. The AT8 epitope (Ser199, Ser202, Thr205) is suggested as a central regulator of cascades that modulate the priming and feedback processes.

2.3 Other molecules interacting with tau

2.3.1 β-Tubulin

Microtubules (MTs) are essential elements in numerous vital functions including cell motility, cell division and cell morphology (e.g. Chau et al. 1998). These functions are regulated by microtubule-associated proteins (MAPs) by stabilizing the MT structure and promoting their dynamics. MTs are non-covalent cytoskeletal tubulin polymers, which are unstable unless stabilized by other molecules (e.g. Kar et al. 2003). These polarized tubulin structures are assembled from α- and β-tubulin monomer subunits forming a heterodimer.

MAP tau binds to β-tubulin subunits within the microtubule through its repeat-region sequences located in the MBD (Lee et al. 1989; Chau et al. 1998; Amos 2004). Upon abnormal hyperphosphorylation, tau loses its function to regulate MT-dynamics leading to disruption of MT-network and impaired axonal transport in AD brain. In addition to tau, β-tubulin, more specificly β-III-tubulin isoform, which is the predominant form of tubulin in neurons, can also be hyperphosphorylated in AD (Vijayan et al. 2001). The decreased PP2A activity in AD brain, which has been also associated with microtubules, reduces tau dephosphorylation and is also suggested to play a part in abnormal phosphorylation of β-tubulin. Although the hyperphosphorylation of β-tubulin may not have a significant impact on MT assembly in young healthy human brain, in AD the contribution of hyperphosphorylated β-tubulin might be noteworthy due to a disturbed tau-related regulation of phosphorylation and MT assembly.

2.3.2 14-3-3ζ

The 14-3-3 family of acidic proteins are regulatory molecules with ability to bind vast array of signalling proteins (Fu et al. 2000). 14-3-3 participates in various cellular processes by binding to its target proteins and thereby regulating and stabilizing enzyme activity and conformation, and, mediates protein-protein interactions and subcellular localization of proteins. Due to the large number of target binding proteins, as an abundant brain protein, 14-3-3 plays a substansial role in many regulatory functions such as cell cycle control, apoptosis and signal

transduction (Fu et al. 2000; van Hemert et al. 2001). To date, seven different isoforms of 14-3-3 is characterized (β, ε, γ, η, σ, τ and ζ), which are all encoded by distinct genes and are expressed in all eukaryotes. These highly conserved phosphoserine-binding proteins largely exist as dimers, with a molecular mass approximately of 30 kDa per subunit (van Hemert et al. 2001; Yuan et al. 2004). Each monomer subunit consists of 9 antiparellel α-helices forming an amphipathic groove that mediates its binding to phosphoserine residues. The regulation of interaction of 14-3-3 with its ligands is highly controlled by diverse mechanisms, such as post-translational modifications, the expression level within the cell and the specifity of different isoforms (Fu et al. 2000).

The phosphorylation status of Ser58 of 14-3-3ζ, which is located within the interface of the dimer, determines whether the 14-3-3ζ is in monomeric or dimeric structure (Woodcock et al. 2003). When phosphorylated at Ser58, 14-3-3ζ is reported to solely exist as a monomer, suggesting that the stability of the dimeric structure is sufficiently disrupted by the phosphorylation of single monomer both *in vitro* and *in vivo*. This specific site has been shown shown to be phosphorylated by protein kinases, such as Akt *in vitro* (Powell et al. 2002) and sphingosine-dependent kinase *in vitro* and *in vivo* (Woodcock et al. 2003). Although the cellular function of 14-3-3ζ is highly facilitated by its dimeric structure, the phophorylation-mediated disruption of the dimer does not inhibit its ability to bind the target proteins indicating a role in substrate activity regulator.

14-3-3 has been strongly indicated to interact with multiple molecules participating in pathways affecting abnormal tau phosphorylation (e.g. Agarwal-Mawal et al. 2003; Hernandez et al. 2004; Kim et al. 2004). Tau, GSK-3β and 14-3-3ζ were reported to be components of a multiprotein complex associated in tau phoshorylation (Agarwal-Mawal et al. 2003). These components have been found to co-immunoprecipitate from brain extracts. The mechanism how 14-3-3ζ connects GSK-3β to tau within the complex remains to be eluciated, but it is suggested that 14-3-3ζ mediates Ser9-phosphorylated GSK-3β and tau interaction, although the interaction between Ser9-GSK-3β and 14-3-3ζ seems to be tau-independent (Yuan et al. 2004). Hence, 14-3-3ζ facilitates the binding of Ser9-GSK-3β to tau and stimulates the phosphorylation of tau by GSK-3β. Additionally, the phosphorylation of tau at Ser214 residue by Akt kinase is reported to highly increase the affinity of 14-3-3ζ towards tau and thereby diminish, or even abolish its aggregation and fibril formation (Sadik et al. 2009).

Upstream of GSK-3β-catalyzed tau phosphorylation, the role of 14-3-3ζ regulating the PKA-mediated phosphorylation of tau has been studied (Hashiguchi et al. 2000; Hernandez et

al. 2004). It has been hypothized that, due to a same binding region in tau and under specific conditions, there may be competion between 14-3-3ζ and tubulin to bind to tau (Hashiguchi et al. 2000). The presence of PKA is shown to reduce the polymerization of tau, due to a presence of 14-3-3ζ, which can facilitate tau phosphorylation and should threfore have decreased ability to form aggregates (Hashiguchi et al. 2000; Hernandez et al. 2004). These results implicate a protecting role of PKA conserning aggregation of tau into fibrillar filaments. There are also findings reporting 14-3-3ζ to increase the activity of DYRK1A, a kinase associated to both AD and DS, through mediating the stability of active structure of 14-3-3ζ (Kim et al. 2004). The binding of 14-3-3ζ to DYRK1A is implicated to be independent of the phosphorylation state of DYRK1A, and, the activation of DYRK1A is suggested to occur in dose-dependent manner *in vitro*.

2.3.3 Pin1

Peptidyl-prolyl isomerases (PPIases) act as a conformatial switch that catalyzes the *cis*/*trans* isomerization of peptidyl-prolyl peptide bonds (figure 2) (e.g. Yaffe 1997; Zhou et al. 1999). There are three highly conserved PPIase-families, cyclophilins, FK506 binding proteins (FKBPs) and parvulins (Lu et al. 1996; Lu, K.P. et al. 2002). Although Pin1 belongs to the subfamily of parvulin PPIases, it is the only PPIase that specifically recognizes and isomerizes phosphorylated serine/threonine-proline sequences (pSer/Thr-Pro) (Yaffe 1997).

Pin1 (protein interacting with NIMA (never in mitosis A) 1) is a ubiquitous enzyme with a high phosphoprotein substrate specifity (e.g. Zhou et al. 1999). Pin1 contains two distinct structural domains; the phosphorylation specific amino-terminal WW domain, which modulates the binding of Pin1 to its substrates, and, the carboxy-terminal PPIase-domain that catalyzes the conformational isomerization of bound substrates (Lu et al. 1996; Yaffe 1997; Lu, K.P. et al. 2002). One of the regulatory mechanisms of Pin1 includes the phosphorylation of Ser16, which is located at pSer/Thr-Pro-binding pocket of Pin1 protein (Lu, P.J. et al. 2002). Ser16 phosphorylation prevents the interaction between WW domain and pSer/Thr-Pro motif, thereby regulating the substrate binding activity. Furthermore, the subcellular localization regulates Pin1 function. The localization of Pin1 within a cell depends on its substrate availability, and in order to bind with a substrate, the interaction with WW domain is required. Hence, the subcellular localization is also dependent on the phosphorylation state of Ser16 of pSer/Thr-Pro motif. Additionally, the expression level of Pin1 regulates its function, which is

normally driven by cell division cycle (e.g. Liou et al. 2002), with an exception in neurons, where the Pin1 level is increased (Lu et al. 1999).

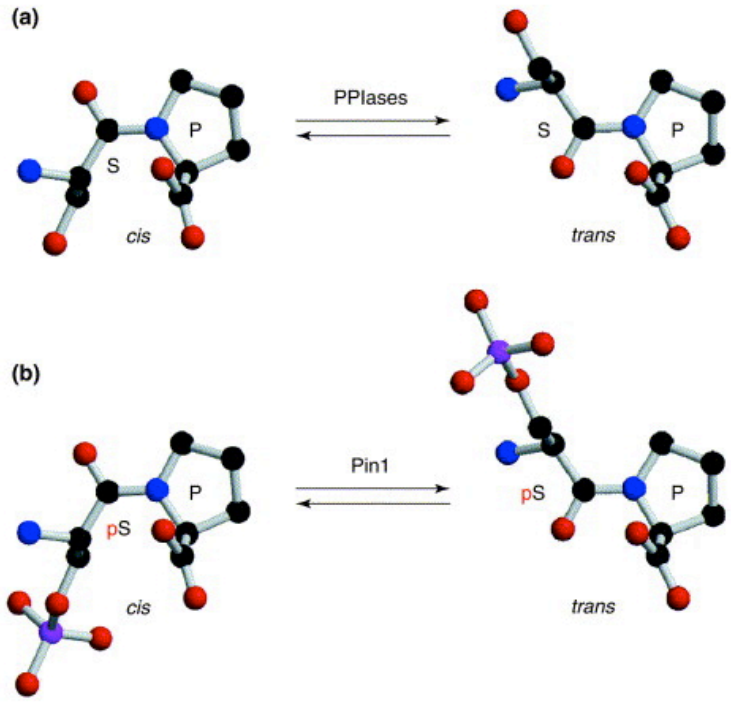


Figure 2. Peptidyl-prolyl *cis/trans* isomerization. The unique property of proline residues to exist in two completely distinct isoforms can potentially provide a switch in the backbone of polypeptide chain (a). This peptidyl-prolyl bond switch is controlled by *cis/trans* isomerization. The spontaneous conversion between the two isomers within peptides is intrinsically a slow process. To avoid these possible rate limitations in protein folding and refolding caused by rather slow spontaneous *cis/trans* isomerization, the conversion is catalyzed by ubiquitous peptidyl-prolyl *cis/trans* isomerases (PPIases). PPIase enzymes are divided in three families; cyclophilins, FK506 binding proteins (FKBPs) and parvulins, which is further divided into subfamilies of parvulin-type and Pin1-type PPIases based on their substrate specifity. Importantly, phopsphorylation of serine/threonine-proline (Ser/Thr-Pro) motifs, which are the major regulatory phosphorylation motifs in the cell, hinders the catalytic action of cyclophilins, FKBPs and parvulin-type PPIases toward these bonds. By contrast, Pin1 and Pin1-type PPIases specifically isomerize phosphorylated Ser/Thr-Pro bonds (b). Isomerization of both phosphorylated and nonphosphorylated Ser/Thr-Pro motifs is highly important due to a conformatial specifity of numerous protein kinases and phosphatases, which phosphorylate and dephosphorylate mostly the *trans*-form of proline bond of proteins. S; serine, P; proline (modified from Lu, K.P. et al. 2002).

Pin1 regulates various cellular processes, due to, at least partially, its wide array of substrates (e.g. Lu, K.P. et al. 2002). These processes include the regulation of cell cycle progression and cellular signalling, modulation of transcription and RNA processing, and, participating in neuronal survival and in responses to DNA damage and cellular stress (e.g. Lu, K.P. et al. 2002; Lu 2004). Under the normal physiological conditions, Pin1 is highly regulated and

the deregulation has been associated with various human diseases, mostly in cancer and AD (e.g. Lu 2004). The finding that Pin1 specifically recognizes and catalyzes the isomerization of pSer/Thr-Pro bonds, which subsequently leads to conformational changes in such motifs in proteins, provided a novel mechanism in post-phosphorylational protein signalling and modification (Ranganathan et al. 1997; Yaffe 1997; Lu 2004).

AD hallmark proteins, APP and tau, are both modified by peptidyl-prolyl *cis*/*trans* isomerase Pin1 (Liou et al. 2003). In the case of APP, it has been suggested that *cis* conformation of phosphorylated Thr668-Pro residue may promote the amyloidogenic processing of APP leading to Aβ formation, and in contrast, *trans* conformation favours the non-amyloidogenic pathway (Pastorino et al. 2006). By catalyzing the conversion from *cis* to *trans* conformation, Pin1 facilitates the non-amyloidogenic APP processing, thereby reducing the Aβ and plaque formation. Interestingly, the amyloidogenic APP processing is reduced by Pin1 mediated GSK-3β inhibition (Ma et al. 2012). By binding to phosphorylated Thr330-Pro motif in GSK-3β, Pin1 inhibits the kinase activity resulting in decreased phosphorylation of Thr668 by GSK-3β and increased turnover of APP. The inhibition of kinase activity by functional Pin1 is reported to occur both *in vitro* and *in vivo* using stable GSK-3β knockdown H4 cells and Pin1-WT, -KO and –Tg mice. Hence, by reducing the total APP levels via increased protein turnover, GSK-3β inhibition by Pin1 provides a novel mechanism to mitigate Aβ-driven AD pathology.

Pin1 binds to and isomerizes tau at phosphorylated Thr231-Pro motif (figure 3) (Lu et al. 1999). Proline-directed PP2A is a conformation-specific phosphatase interacting only with the *trans* isomeric form of pSer/Thr-Pro motif (Zhou et al. 2000). Hence, by catalyzing the conversion from *cis* to *trans* isomer, Pin1 facilitates the dephosphorylation of tau by PP2A and may restore the ability of tau to bind and stabilize microtubules (Lu et al. 1999; Zhou et al. 2000). Depletion of Pin1, or its decreased activity as seen in AD, induces the *cis* isomer of pThr231-Pro motif to accumulate, leading to increased tau hyperphosphorylation and formation of neurofibrillary tangles. In addition, the dephosphorylation on Ser16 residue of Pin1 and simultaneous dephosphorylation of tau at Thr231 residue, is suggested to be induced by Aβ (1-42) treatments in hippocampal neurons (Bulbarelli et al. 2009). These results suggest that tau hyperphosphorylation may be reduced or prevented due to an Aβ (1-42) induced Pin1 function. Taken together, Pin1 has an essential role in protection against age-dependent neurodegeneration and provide a noteworthy candidate to targeted AD treatment (e.g. Liou et al. 2003).

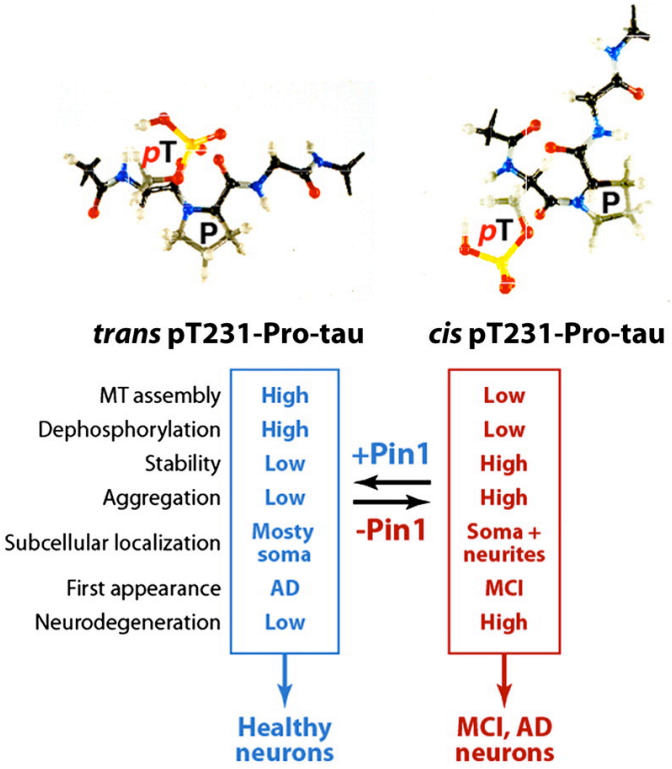


Figure 3. Pin1 catalyzed *cis/trans* isomerization of Proline-directed pThr231-tau in Alzheimer's disease. Phosphorylated threonine 231 residue in tau can adopt into two completely distinct conformations (*cis* and *trans*), a conversion catalyzed by Pin1 between the two conformations. Pathogenic *cis*-form may result in loss of normal microtubule stabilizing function of tau and further induce toxic gain-of-function. Acting as a conformatial switch by converting pThr231-tau from pathogenic to nonpathogenic conformation, Pin1 prevents the accumulation of the more stable *cis* form. This may subsequently restore the normal tau functions such as proper subcellular localization and decrease aggregation, and promote the dephosphorylation of tau in Alzheimer's disease. MCI; mild cognitive imparment (modified from Nakamura et al. 2012).

2.4 Other post-translational modifications

Besides phosphorylation, tau protein can be post-translationally modified through various mechanisms (e.g. Gong et al. 2005). These modifications include glycosylation, glycation, ubiquitination, polyamination, truncation, nitration (Gong et al. 2005) and acetylation (Min et al. 2010). Both, under the normal and under the pathological conditions, these post-translational modifications plays a pivotal role in converting the normal functional tau towards PHF/SF- formation and subsequent neurofibrillar degeneration in AD and related tauopathies (Gong et al. 2005; Wang & Liu 2008).

In glycosylation, oligosaccharides are covalently linked to protein side chains, in a reaction that is facilitated by various enzymes such as glycotransferases (Gong et al. 2005; Wang &

Liu 2008). Depending on the form of the glycosidic bond, two distinct glycosylation types have been identified: O-linked and N-linked glycosylation. N-glycolysation defines a reaction, in which the oligosaccharides are covalently linked to the amino groups of asparagine side chains. Protein N-glycosylation normally takes place in rough endoplastic reticulum and Golgi apparatus (e.g. Gong et al. 2005), and is enhanced in PHF-tau in comparison to normal functioning tau (Liu et al. 2002b). It has been reported that abnormal glycosylation promotes tau hyperphosphorylation by activating phophorylation via specific kinase pathways (e.g. PKA, GSK-3β and CDK-5) and by inhibiting dephosphorylation by PP2A and PP5 (Liu et al. 2002a). Additionally, the effects of glycosylation on phosphorylation are site-specific, and, aberrant glycosylation precedes abnormal tau hyperphosphorylation (Liu et al. 2002a; Gong et al. 2005).

In O-glycosylation, oligosaccharides are added to serine or threonine residues of hydroxyl groups that are in close proximity of proline residues (Gong et al. 2005; Wang & Liu 2008). Especially, the addition of O-linked monosaccharide β-N-acetylglucosamine (O-GlcNAc) to serine/threonine residues of tau has been studied (Arnold et al. 1996; Liu, F. et al. 2004). O-GlcNAcylation has been found to be decreased in AD brains, which is at least partially due to the observation, that O-GlcNAcylation occurs in same serinene/threonine sites with phosphorylation in tau (Liu, F. et al. 2004). Moreover, an impaired brain glucose metabolism negatively regulates O-GlcNAcylation, which may lead to consequent hyperphosphorylation of tau. Although the glycosylation is one of the most common post-translational modifications of tau, the molecular mechanism(s) is yet to be determined (Gong et al. 2005). However, the down-regulation of O-GlcNAcylation provides one mechanism by which defective brain glucose metabolism is linked to development of AD.

In a very recent study, it was shown that chemical inhibition of glycoside hydrolase (O-GlcNAcase), which removes the O-GlcNac from the Ser/Thr residues, increased the amount of O-GlcNAc and effectively slowed the loss of motor neurons *in vivo* (Yuzwa et al. 2012). The O-GlcNAcase was inhibited in JNPL3 mice, a transgenic mouse model that expresses human FTDP-17 tau P301L mutant transgene and develops neurofibrillary tangles, using Thiamet-G as an inhibitor and resulted in decreasing number of NFTs compared to vehicle treatments. Interestingly, the modification of O-GlcNAcylation did not alter the phosphorylation status of the sites studied *in vitro*, which suggests that the mechanism is completely phosphorylation-independent. By using various tau mutants, the O-GlcNAcylation of Ser400 residue was reported to play a major role in decreased tau fibrillization. Furthermore, no apparent adverse side effects were reported in these mice over a treatment period of 8 months.

Importantly, the protective effect of O-GlcNAcylation against protein aggregation does not seem to be only specific for tau, but also could hinder the aggregation and promote the stability of totally unrelated proteins. Hence, the modification of O-GlcNAcylation could provide a novel candidate for therapies for tauopathies and preventing the aggregation of unrelated proteins and stabilize their cellular function.

Different from enzymatic glycosylation, glycation denotes non-enzymatic linkage of reducing saccharides to amino side chains of polypeptides (Yan et al. 1994; Gong et al. 2005). Advanced glycation end products (AGE) are heterogenous formations of subsequent oxidation of protein glycation, which are shown to be present in PHFs/NFTs (Yan et al. 1994; Sasaki et al. 1998). Furthermore, tau glycation was shown to induce neuronal oxidative stress (Yan et al. 1994) and has also been suggested to facilitate the formation of PHFs (Kuhla et al. 2007).

Unlike normal tau and soluble abnormally hyperphosphorylated tau, PHF-tau and NFTs are polyubiquitinated in AD (Perry et al. 1987). Misfolded or damaged proteins under normal conditions are ubiquitin-labelled and degraded by the ubiquitin-proteasome pathway in an ATP-dependent manner (e.g. Gong et al. 2005). However, the degradation of polyubiquitinated PHF-tau is highly reduced in AD brains, which may be due to an impaired proteasome function, leading to concurrent aggregation of tau and to formation of NFTs (Keller et al. 2000). Additionally, another modification affecting the degradation rate of tau is polyamination (Tucholski et al. 1999). Results from *in situ* studies suggests that the addition of polyamines to tau in a reaction catalyzed by tissue transglutamase, does not largely compromise its microtubule binding affinity, but exacerbates the calcium-induced degradation by calpain protease.

The C-terminal truncation of tau by caspase and other proteases changes the conformation of tau and, compared to normal full-lenght form, cleaved tau is more prone to aggregate into PHFs/NFTs (Yin & Kuret 2006). Truncation of tau is suggested to occur at early stages of disease development of AD, and also, that it can induce the tau fibrillization even in low concentrations, i.e. the critical consentration is lowered due to C-terminal tau truncation.

The nitration of tau has been reported to contribute to tau filament formation and it has been shown to co-localize with NFTs in AD (Horiguchi et al. 2003). This post-translational modification of tau is site-specific towards tyrosine residues Tyr18, Tyr29, Tyr197 and Tyr394 (Reynolds et al. 2005), from which Tyr29-nitrated tau is reported the most affected in AD brains, and therefore suggested to have an impact on AD progression (Reynolds et al. 2006). Furthermore, it has been recently reported that tau is acetylated, and, that acetylation of

tau prevents the ubiquitin-mediated degradation of abnormally hyperphosphorylated tau (Min et al. 2010). This study suggests the role of histone acetyltransferase p300 as a promoter of tau acetylation, and in contrast, a protein deacetylase SIRT1 to induce the deacetylation. Moreover, SIRT1 deprivation is suggested to promote tau hyperacetylation, which may result in abnormal hyperphosphorylation and neurofibrillar degeneration. This effect was shown to be reversed by inducing the deacetylation of tau.

After the reversible acetylation of tau emerged as a novel post-translational modification, its molecular mechanism and significance on tau function in AD and other neurodegenerative tauopathies has been of interest in multiple studies. Interestingly, the lysine residue 280 (K280) within a microtubule-binding region of tau pointed out to be the major target site of acetylation and it may have a pathological role in AD and other tauopathies (Cohen et al. 2011). Due to its localization within the microtubule-binding motif, acetylated K280 residue disrupts the normal tau function by preventing its binding to MTs resulting in decreased MT-assembly and stability (Cohen et al. 2011; Irwin et al. 2012). In addition to loss of normal tau functions, toxic gain of functions such as increased tau fibrillization and increased amount of soluble tau prone to oligomerization to PHFs uniformly contribute to tau-driven neurodegeneration. Thereby, inhibition of acetylation of tau K280 could offer a novel candidate for drug discovery for tauopathies. Nevertheless, the connection between phosphorylation and acetylation in specifically identified multiple sites and their dynamics and effects on tau oligomerization need to be further investigated to fully understand the synergism of these post-translational modifications.

2.5 Role of tau in central nervous system disorders

Abnormally hyperphosphorylated tau is the predominant protein subunit forming PHFs/SFs in Alzheimer's disease brain (e.g. Grundke-Iqbal et al. 1986; Iqbal et al. 1989). The subsequent formation of neurofibrillary tangles is the second neuropathological hallmark lesion of AD in addition to amyloid plaques. It has been established that the NFTs in AD, and not the Aβ plaques, are the lesions that correlates with the severity of cognitive decline and dementia (e.g. Buee et al. 2000; Wang & Liu 2008). It seems that amyloid plaque pathology precedes NFT pathology in AD. Regardless of AD being the most common and the most studied sporadic tauopathy, it is not fully understood how abnormally hyperphosphorylated tau and the formation of filaments affect on the learning impairment and memory deficits.

The aggregation of abnormally hyperphosphorylated tau is not specific to AD, but has been described in several other neurodegenerative disorders referred as tauopathies (table 2) (e.g. Buee et al. 2000; Iqbal et al. 2005). The development of each of these tauopathies involves neurofibrillary degeneration associated with dementia, which is induced by aberrant accumulation of hyperphosphorylated tau. The mechanisms that may promote the alterations in tau metabolism includes missense mutations, various post-translational modifications such as phosphorylation and glycosylation, altered expression of different tau isoforms or the amount of total tau expression (e.g. Hernandez & Avila 2007). The discoveries of several tau gene missense mutations that co-segregate with the disease in Frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17), implicates that tau abnormalities *per se* are disease-causative and could lead to neurofibrillary degeneration (e.g. Hutton et al. 1998; Spillantini et al. 1998). These findings together with the notified absence of tau mutations in AD, emphasizes the critical role of tau in AD pathogenesis and that tau abnormalities can directly be disease-causative (e.g. Brunden et al. 2008).

In FTDP-17, one of four known tau missense mutations (G272V, P301L, V337M and R406W) results in tau forms that are *in vitro* better substrates to be phosphorylated by various protein kinases in the brain compared to wild-type human tau (Alonso Adel et al. 2004). Moreover, these mutated tau proteins are more rapidly and with higher extent hyperphosphorylated than 4R-taus, which in contrast hyperphosphorylates faster than 3R-tau species. The self-aggregation of mutated tau species requires 4-6 moles of phosphate per mole of tau to form filaments, whereas ~10 moles of phosphate per mole of protein is required in the case of wild-type tau to polymerize. The conformational change in tau induced by these missense mutations, besides making it more prone to hyperphosphorylation, also promote the sequestration of normal tau, which could further explain the severity and autosomal dominance of inherited FTDP-17. To date, 32 different mutations including missense, deletion, silent and intronic mutations have been described in more than 100 FTDP-17-families (Goedert & Jakes 2005). From these mutations, approximately half contributes to increased abnormal filament formation of tau and its reduced ability to interact and bind to microtubules. The other half of the mutations affects the RNA level of tau by disrupting the normal 4R/3R-ratio that has been observed in various neurodegenerative disorders.

Normally the ratio of 4R-tau to 3R-tau isoforms in healthy human brain is approximately 1 (Hong 1998). In FTDP-17, the ratio of 4R/3R is dramatically increased mainly due to the alternative splicing of exon 10 induced by intronic mutations (e.g. Hutton et al. 1998; Spillantini et al. 1998). The findings that neurofibrillay tangles isolated from affected areas of AD

brain shows increased amount of 4R-tau (e.g. Glatz et al. 2006), and, that the 4R-tau is the predominant isoform in tau polymers in FTDP-17 (e.g. Hong 1998), may at least partially explain why the increase in 4R/3R ratio can cause neurofibrillary degeneration (e.g. Iqbal et al. 2005; Wang & Liu 2008). On the contrary, in Pick's disease and Down syndrome, both characterized by early onset dementia, 3R-tau (lacking exon 10) is the major isoform expressed (Yoshida 2006; Shi et al. 2008). Therefore, according to the contradictive manifestations of both 4R-tau and 3R-tau in various neurodegenerative diseases, it seems that it is more likely the altered ratio of these isoforms rather than the toxicity of each tau isoform *per se* that is promoting neurofibrillary degeneration in tauopathies (e.g. Wang & Liu 2008).

Table 2. Neurodegenerative tauopathies involving abnormally hyperphosphorylated tau. In some of these diseases abnormal tau inclusions are the most predominant neurodegenerative brain lesions (*) (modified from Gong et al. 2005).

Disease
Alzheimer's disease (AD)
Argyrophilic grain dementia*
Bliut disease
Corticobasal degeneration*
Dementia pugilistica*
Diffuse neurofibrillary tangles with calcification*
Down's syndrome (DS)
Frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17)*
Gerstmann-Sträussler-Scheinker disease
Guam parkinsonism dementia complex*
Multiple system atrophy
Myotonic dystrophy
Neurodegeneration with brain iron accumulation type 1 (Hallevorden-Spatz disease)
Niemann-Pick type C disease
Pick's disease*
Post-encephalitic parkinsonism
Prion diseases
Progressive subcortical gliosis*
Progressive supranuclear palsy*
Subacute sclerosing panencephalitis
Tangle only dementia*

3 PCA AS A METHOD TO STUDY PROTEIN-PROTEIN INTERACTIONS

Exploring prorein-protein interactions is crucial to understand biochemical pathways in a living cell (Michnick 2001). Molecular pathways are networks consisting of protein complexes simultaneously assembling and disassembling hereby eliciting highly dynamic biochemical networks. Different strategies to investigate these interactions have been developed in order to

understand and define the function and organization of the genes, their products and complex biochemical networks (Michnick et al. 2007). Protein-fragment complementation assay (PCA) is one strategy to study the dynamics and kinetics of interacting proteins in the living cell. One of the key points when designing PCA fragmets is to try to prevent spontanious folding of the reporter protein fragments (Michnick et al. 2000). If spontanious folding cannot be prevented, the false-positive signal could confound the interpretation of the data received from protein-protein interactions.

PCA is a method to investigate protein-protein interactions based on a reconstitution of enzyme activity of a reporter protein (Michnick 2001). In PCA, two complementary fragments of a reporter protein are both fused with two proteins of intrest. Due to an all-or-none nature of protein folding, when these fusion proteins interact, folding of a reporter protein to its native conformation restores the enzyme activity, which can be detected (figure 4). Therefore, in case the proteins of interest are not interacting, no signal can be detected.

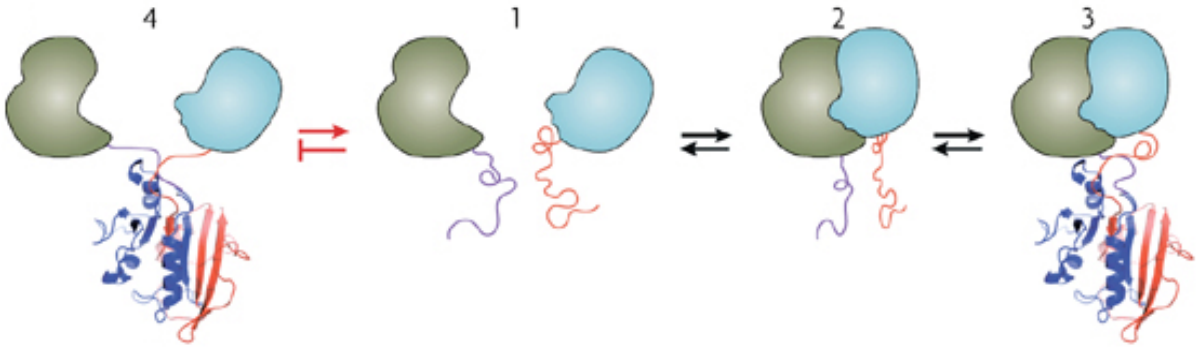


Figure 4. The principle of protein-fragment complementation assay (PCA). In the PCA, interacting proteins of interest are fused to the two complementary fragments of a reporter protein (e.g. humanized *Gaussia princeps* luciferase, hGluc) (red and blue). Protein folding in a PCA is an all-or-none process. When the two proteins of interest interact in a cellular environment the unfolded reporter-protein fragments are brought into close proximity allowing them to fold into their active native conformation (1→2→3). The restoration of the enzyme activity enables the measurement of specific PCA reporter signal (e.g. luminescence). Depending on the endpoint application of proteins of interest (e.g. localization or quantification), the PCA-reporter protein could be selected based on its features, for example reversibility or irreversibility of the reporter dynamics (hGluc or green fluorescent protein, respectively) (modified from Michnick et al. 2007).

PCAs based on photoproteins as reporters are highly useful for studying protein-protein interactions (Tannous et al. 2005). Photoproteins, such as fluorescent and bioluminescent proteins, have diverse properties, which can be used to investigate various phenomena in live-cell environment. Fluorescent proteins (e.g. green fluorescent protein (GFP) and YFP) are useful in trapping and visualizing more rarely occurring protein complexes due to irreversibility of

refolding of splitted fluorescent reporter protein (e.g. Hu et al. 2002). Furthermore, using fluorescent proteins have been proved as a useful method to study the relocalization of complexes using small molecules to perturb the normal balance (Michnick et al. 2007). Irreversible protein folding is a general concern in PCAs by preventing the disassociation of the protein complex, hereby preventing the studies of protein-protein kinetics or dynamics performed in the time frame of seconds or minutes. Unlike fluorescent-based PCAs, bioluminescence-based PCAs have been proved to be completely reversible (Remy & Michnick 2006).

Bioluminescent proteins possess many beneficial properties concerning their use as receptor proteins in PCAs (Remy & Michnick 2006). A class of bioluminescent proteins called luciferases catalyze an ATP-independent reaction where light is emitted (at a peak of 480 nm) due to substrate oxidation (Tannous et al. 2005). Luciferase enzyme, which is isolated from marine copepod *Gaussia princeps*, is composed of 185 amino acids (19.9 kDa) and uses coelenterazine as a substrate. The humanized form of monomeric protein *Gaussia* luciferase (hGLuc) generates over 100-fold bioluminescense signal compared to other humanized forms of luciferases, such as *Photinus pyralis* (firefly, hFLuc) and *Renilla reniformis* (hRLuc), in cell lysates or in living cells. hFLuc differs from hRLuc and hGLuc by its different substrate (beetle D-luciferin) and its dependency on cofactors (ATP and Mg^{2+}) for activity (Lembert & Idahl 1995). In addition, hFLuc has slow glow kinetics in contrast to rapid flash kinetics of hRLuc and hGLuc (Tannous et al. 2005). The substrate coelenterazine allows quantative analysis and real time monitoring of the conversion reaction through its ability to permeate cell membranes and thereby to diffuse into all cellular compartments (Remy & Michnick 2006). Since being the smallest of all known coelenterazine-using luciferases, hGluc has great potential for diverse uses (Tannous et al. 2005).

Regardless of the receptor protein used, PCA has many advantages studying protein-protein interactions, whether concentrating on localization, quantification or investigating the dynamics of the proteins of intrest (Michnick et al. 2007). Particularly, perturbation of interactions by genetic (gene overexpression or knockdown) or pharmacological means is an important and efficient strategy to study the alterations of specific biochemical pathways. Moreover, PCAs can be quite easily automated for high-throughput screening (HTS) to search for novel drug candidates and are also inexpensive. Split-GFP and split-luciferase PCAs both have specific investigation targets albeit they share at least some common features (table 3). The most crucial benefits with hGLuc-based PCA is the high sensitivity in real time detection of *in vivo* or *in vitro* studies and the ability to study the kinetics of assambly and disassambly of protein complexes in living cells (Remy & Michnick 2006). Although the method has

many advantages, it also has a few limitations. hGLuc PCA requires overexpression of reporter protein constructs which is always an aberration compared to normal condition of cell environment. Also, the addition of substrate is required to achieve an active reaction to induce bioluminecense, while fluorescent reporter proteins do not have this requirement.

Table 3. Features of different methods to study protein-protein interactions. eBRET: Extended bioluminescence resonance energy transfer; FRET/FLIM: Förster (fluorescence) resonance energy transfer/fluorescence lifetime imaging microscopy; GFP: green fluorescent protein; hGLuc: humanized *Gaussia princeps* luciferase.

Method	**Live cells**	**Localization**	**Quantification**	**Dynamics**
Immunoprecipitation	-	-	+ +	-
eBRET	+ +	-	+ +	+
FRET/FLIM	+ +	+ +	+ +	-
Split fluorescent protein PCA (e.g. GFP)	+ +	+ +	+	-
Split bioluminescence protein PCA (e.g. hGLuc)	+ +	-	+ +	+ +

4 AIMS OF THE STUDY

Tau protein is one of the aggregating proteins in various neurodegenerative disorders such as Alzheimer's disease and frontotemporal dementia. Dissociation of tau from microtubules and its intracellular aggregation is regulated by phosphorylation and dephosphorylation equilibrium. Studying the alterations of tau phosphorylation within live cell environment using small molecular compounds to induce or inhibit biochemicals pathways, could offer a novel method to study and further to understand the mechanism(s) of tau-driven neurodegeneration. Aims of the study were to

- set up and validate PCA to investigate protein-protein interactions of tau in live cells
- validate the conditions and perform high-throughput screening
- confirm the possible hits from the screen using independent methods.

5 MATERIALS AND METHODS

5.1 Cloning

All the cDNAs used in cloning were purchased from MGC IMAGE cDNA library (Open Biosystems). Inserts were amplified from cDNAs using PCR with specifically designed primers (Oligomer) and Phusion Hot Start DNA polymerase enzyme (Finnzymes) (table 4). Each PCR-program was determined separately considering the melting temperatures (Tm) of the

primers used. PCR-products were cut out from the 1% agarose gel after gel electrophoresis and were purified using QIAquick Gel Extraction kit (Qiagen). Purified insert DNAs were digested with various restriction enzymes (New England Biolabs, NEB) by single enzyme digestion and the DNA fragments were purified (MiniElute PCR Purification Kit from Qiagen) before second digestion. All restriction enzyme digestions were incubated 2 hours in 37°C. After the second digestion inserts were purified (MiniElute PCR Purification Kit from Qiagen) and the concentrations were measured using NanoDrop 2000c spectrophotometer (Thermo Scientific). DNAs were eluted in Milli-Q water (mQH_2O).

Table 4. Primers (Oligomer) used in PCR-cloning of the constructs. Cdk5-construct was cloned by Prasanna Sakha. Primers HH-0004 and HH-0024 were used in colony PCR and in sequencing (HH-0004).

Primer	Sequence	Tm	Gene
HH-0009B	ATCGATGGGCGGACAGAAGTCGGA	61°C	hCdk5
HH-0009C	GATATCACCGCCATGCAGAAATACGAGA	59°C	hCdk5
HH-0013C	GAAGATCTACCGCCATGTCAGGGC	59°C	hGSK3β
HH-0013D	TGAATTCGGTGGAGTTGGAAGCTGATG	59°C	hGSK3β
HH-0015F	CGGGATCCACCGCCATGGCTGAGCCCCG	71°C	hTau
HH-0015G	TGAATTCCAAACCCTGCTTGGCCAGGGAGG	66°C	hTau
HH-0017A	GCGGCCGCACCGCCATGGCAGGAGCT	73°C	hPPP2R2A
HH-0017B	ATCGATATTCACTTTGTCTTGAAATATATACAG	53°C	hPPP2R2A
HH-0018A	GCGGCCGCACCGCCATGGATAAAAAT	65°C	h14-3-3z
HH-0018B	ATCGATATTTTCCCCTCCTTCTCCTGCTTC	60°C	h14-3-3z
HH-0026D	GAAGATCTACCGCCATGCATACAGG	57°C	hDYRK1A
HH-0026E	TGAATTCCGAGCTAGCTACAGGACTC	58°C	hDYRK1A
HH-0034C	CGGGATCCACCGCCATGGGCTGTG	66°C	hFyn
HH-0034D	TGAATTCCAGGTTTTCACCAGGTTGGTACTGGGG	65°C	hFyn
HH-0048A	CGGGATCCACCGCCATGGCGGAC	66°C	hPin1
HH-0048B	TGAATTCCTCAGTGCGGAGGATGATGTGG	62°C	hPin1
HH-0004	TAGAAGGCACAGTCGAGG	50°C	pcDNA3.1
HH-0024	TAATACGACTCACTATAGGG	45°C	pcDNA3.1

Constructs phGLuc1-APP and phGLuc2-APP, which were kind gifts from Oksana Berezovska (Massachusetts General Hospital, Boston, USA) with permission of Stephen W. Michnick (Université de Montréal, Montréal, Canada), were used as template plasmids for further modifications of phGluc-vectors. APP inserts were removed from the vectors by restriction enzyme (RE) digestions and the vectors were blunted using NEB Quick Blunting Kit (according to manufacturer's protocol). After purification, self-ligation was performed for both blunted vectors using T4 DNA ligase (NEB) in room temperature (RT) for over night (o/n). Electroporation was used for transformation of plasmids to XL1 Blue electroporation competent cells (E. coli). In electroporation, 1 µl of self-ligated plasmid was pipetted to thawed XL1 Blue cells on ice, gently swirled and transfered into ice-cold cuvette. Electropo-

ration was proceeded using 2500 V for 0,5 milliseconds according to manufacturer's protocol for high efficiency electrotransformation of E. Coli (MicroPulser, Bio Rad). Transformated cells were plated on LB-plates with suitable antibiotic after 1 h (37°C, shaker) incubation in 1 ml of SOC media. Plates were incubated o/n in 37°C and checked for colonies. These resulted vectors containing more flexible multiple cloning sites (MCS), named phGluc(1C) and phGluc(2C), were used for cloning C-terminal hGluc fusion constructs (figure 5).

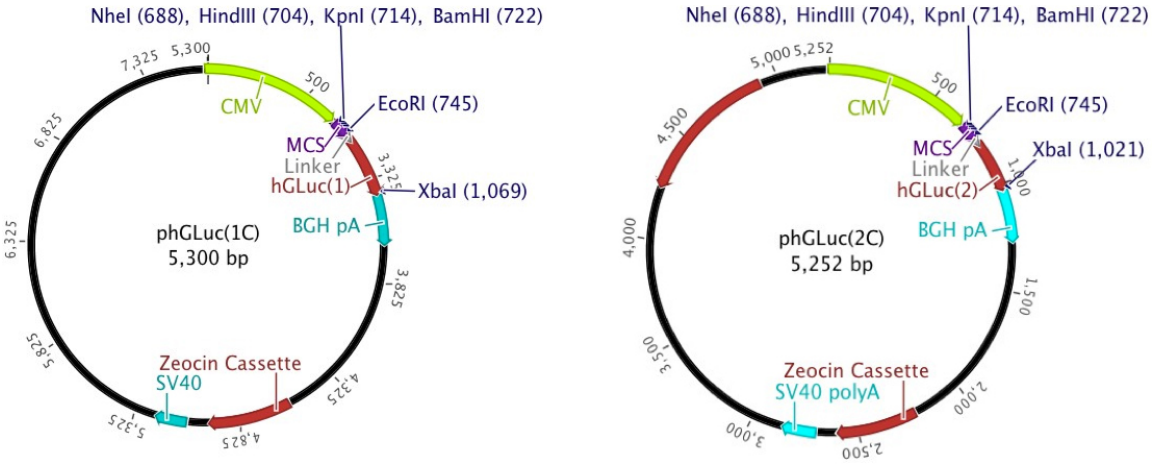


Figure 5. Vectors phGluc(1C) and phGluc(2C). Plasmids were modified from original plasmids by removing the inserts, blunting and self-ligating the vector backbone. These vectors were used in most of the clonings of constructs that were further used in PCA. Vectors were digested at multiple cloning sites (MCS) with various restriction enzymes and ligated with identically digested inserts.

Inserts and vectors were ligated using T4 DNA ligase (NEB) and 10X T4 ligation buffer (NEB) in 10 µl reactions which were incubated o/n in 12°C and transformed the following day to XL1 Blue cells (1 µl of ligation mix) using electroporation. Clones containing the insert were screened using colony-PCR or by restriction enzyme digestions from miniprep DNA. Minipreps were produced by inoculating the colony into 3 ml of LB-medium (Luria-Bertani medium) with suitable antibiotic which after o/n incubation in 37°C on shaker were purified with QIAprep Spin Miniprep Kit (Qiagen). Verifications were done by using RE digestions and gel electrophoresis. Colony-PCR screening was used when screening large number of colonies using Dynazyme II DNA polymerase (Finnzymes) and specifically designed primers (one primer having recognition site in the vector backbone, and the other primer in the insert). All positive clones were verified by sequencing (Institute of Biotechnology, University of Helsinki, Helsinki, Finland).

5.2 Cell culture and transfections

Mouse neuroblastoma (Neuro2A) cells were maintained at 37°C, 5% CO_2 in fully supplemented Dulbecco's Modified Eagle's Medium (DMEM (Lonza) including 10% fetal bovine serum (FBS; Lonza), penicillin/streptomycin (Lonza) and L-Glutamine (Lonza)). Morphology and density of Neuro2A cells were monitored with microscope and the cells were passaged twice a week. Rat cortical neurons (RCN) were maintained in same conditions as Neuro2A, except that fully supplemented neurobasal medium (NB including 2% B27 (Invitrogen), 1% penicillin/streptomycin and 1% L-Glutamine) was used. RCNs were plated on Poly-L-lysine (Sigma) (1:10 diluted in mQH_2O) coated 6-well plates in density of 400 000 cells per well in 2 ml of full NB. 1/3 of the media was changed twice a week and due to an evaporation 100µl more media was added to the wells than pipetted out of the wells to maintain the total volume of 2 ml per well.

For transfections of Neuro2A cells plated on a Poly-L-lysine coated (Sigma) (1:10 diluted in mQH_2O) white walled clear bottom plates (ViewPlate -96 TC, PerkinElmer), 8 000 or 10 000 cells per well in 200 µl of full DMEM, were carried out using jetPEI™ transfection reagent (Polyplus Transfection) according to manufacturer's protocol. 1:3 ratio of DNA: jetPEI was used (0,1 µg of DNA, 0,3 µl of jetPEI per well) diluted in suitable volume in 150 mM NaCl (parameters are for 96-well plate transfections). Cells were transfected 24 h post-plating and incubated in 37°C, 5% CO_2. The validation of transfection efficiency and the selection of cell line used were done by Prasanna Sakha (Neuroscience Center, University of Helsinki, Helsinki, Finland).

5.3 Immunofluorescence microscopy

Neuro2A cells were plated on a Poly-L-lysine coated 6-well plate containing glass coverslips (3×9mm coverslips per well) at the density of 200 000 cells per well in 2 ml of fully supplemented DMEM. Cells were transiently transfected 24 h post-plating as described above using pEGFP-tubulin and phGluc(2C)-tau plasmids at ratio of 1:1. 24 h post-transfection the cells were fixed with 3% paraformaldehyde in PBS for 20 min and washed 3×2 min with PBS. Then, the coverslips were incubated in the blocking buffer (5% goat serum, 1% bovine serum albumin (BSA), 0,1% gelatin, 0,1% Triton X-100, 0,05% Tween-20 in PBS) for 1 h at RT. Next, the coverslips were incubated with the primary antibody Tau-5 (Invitrogen) diluted 1:500 in primary antibody dilution buffer (1% BSA, 0,1% gelatin in PBS) at +4°C with gentle shaking over night. After washing the cells 3×3 min with PBS, Alexa Fluor-conjugated (568)

Goat anti-mouse secondary antibody (Molecular Probes/Invitrogen) was diluted 1:2 000, added to coverslips and incubated for 45 min, RT, shaker (protected from the light). To counterstain the nuclei, the cells were again washed 3×3 min with PBS and then incubated with 1:10 000 diluted Hoechst 33342 (Molecular Probes/Invitrogen) in PBS for 10 min following the washes of 2×2 min with PBS and once with mQH_2O. Finally, the coverslips were mounted on microscope slides with ProLong Gold antifade reagent (Molecular Probes/Invitrogen), dried for 30 min and sealed on the microscope slides using nail polish. Microscope images were taken using epifluorescence microscope (Zeiss Axio Imager M1), which was equipped with a CCD camera (AxioCam HRm CCD).

5.4 Protein-fragment complementation assay

Protein-fragment complementation assays (PCA) were performed using Neuro2A cells. 8 000 cells were plated on a 96-well plate in 200 µl of fully supplemented DMEM and transfected 24 h post-plating as described. Prior to starting the treatments the wells were washed once with pre-warmed 1 × PBS (phosphate bufferd saline) after discarding the medium/transfection -mix from the wells, either by pipetting or flipping and tapping the plate. All the compounds used in the treatments were diluted to phenol-red free DMEM (PRF-DMEM, Invitrogen) containing penicillin/streptomycin and without FCS and L-glutamine, which was also added to the cells to be left untreated (controls). The volume of the medium used in the incubations was 75 µl per well. Incubation times with treatments on varied from 1 h to 4 h and a minimum of 4 replicate wells of each treatment (or untreated) was used.

The timing of treatments was planned so that the measurement of bioluminescence was performed 48 h post-transfection. The luciferase substrate used, native coelenterazine free base (nCol, NanoLight Technology), was dissolved in methanol (1 mg/ml) and further diluted into PRF-DMEM (80 µM). Immediately after well-by-well injection of 25 µl of nCol per well, resulting the final concentration of 20 µM nCol, the luminescence was measured (Victor^3 1420 Multilabel counter with dispenser unit, PerkinElmer). Various measurement protocols were used during the validation of PCA from which the protocol consisting of 1 second measurement repeated 5 times with 0,2 second interval in between was the mostly used. Avarage of the five 0,2 s readings was used as the reading per well.

5.5 High-throughput screening

High-throughput screening (HTS) was carried out in cooperation with Dr. Päivi Tammela from Centre of Drug Research (CDR, University of Helsinki, Helsinki, Finland) using CDR's libraries of pharmaceutical compounds (PC) (appendix 1) and natural compounds (NC) (appendix 2). PCs and NCs were pre-diluted in dimethyl sulfoxide (DMSO) on 96-well master plates (10 mM and 20 mM, respectively) by Dr. Päivi Tammela. The interaction pair selected and used in the HTS was tau-Pin1.

The sample plates were prepared manually from master plates by diluting the pre-diluted stock solutions to desired concentrations in PRF-DMEM. At the primary screening round the concentration used for compounds was 50 μM, 4 replicate wells each. Hence, one assay plate contained 20 different compounds to be tested and untransfected-, 5 μM Juglone- and 25 mM KCl controls, 4 replicate wells each. The first screening round included the whole PC/NC library containing altogether 355 compounds (240 and 115, respectively). On the secondary screening round, 5 different concentrations (50 μM, 25 μM, 12,5 μM, 6,25 μM and 3,125 μM, 4 replicate wells each) were used for selected compounds (15 PCs, fresly dissolved) to verify results from the first round. Based on the observations from the second round, the third screening round was performed using logaritmic concentrations (50 μM, 5 μM, 0,5 μM, 0,05 μM and 0,005 μM) for further selected compounds (7 PCs and 5 NCs).

The plating of Neuro2A cells (10 000 per well) and transfections for HTS were performed as described. 46 h post-transfection the assay plates were washed once with warm (37°C) 1 × PBS semi-automatically by tilting the plate over and tapping the plate gently against paper towel to empty the wells (DMEM and transfection mix), added the 1 × PBS using robot (Beckman Coulter Biomek FX Dual-pod workstation) and discarded the PBS as described. Robotics was also used for addition of compounds from pre-warmed sample plates to assay plates. Treatment time was 2 h. The measurements were performed using Varioskan Flash (Thermo Scientific) using measurement protocol; 0,2 second measurement repeated 5 times per well without pause in between. The luminescence was measured well-by-well immediatly after the injection of the substrate.

5.6 Western blotting

Proteins were extracted from 21 days *in vitro* (DIV) rat cortical neurons (RCNs) after 2 h treatments (also 6 h and 24 h treatments were tested) using ice-cold full extraction (GTIP) buffer (for 10 ml: 1 ml 10 × GTIP buffer (100 mM Tris pH 7,6, 20 mM EDTA and 1,5 M

NaCl), 25 µl NP40 (Igepal CA-630, Sigma), 1% TritonX-100 (Sigma), 1 pill of complete Mini-EDTA free (protease inhibitor cocktail, Roche), 1 pill of PhosStop (phosphatase inhibitor cocktail, Roche), 100 µl 0,1M NaF and 8 ml mQH_2O) (table 5). The volume of full NB of each well was measured and adjusted to 2 ml the previous day before the treatments. Protein concentrations of the cell lysates were determined using BCA Protein Assay (Pierce/Thermo scientific) and the samples were prepared in the total volume of 40 µl including 4 × gel-loading buffer (NuPAGE LDS buffer, Invitrogen) containing β-mercaptoethanol. Samples and standards (Precision Plus Protein Kaleidoscope, Bio-Rad) were loaded into NuPage 4-12% Bis-Tris Gels (Invitrogen) and the gels were run using XCell SureLock Mini-Cell (Invitrogen) containing 1 × MES SDS Running buffer (170 V, 60 min).

Table 5. Controls and pharmaceutical compounds (PC) used in RCN treatments. The final concentration for all the PCs used was 50 µM which were added directly into the media (fully supplemented neurobasal) and incubated in 37°C, 5 % CO_2 for 2 h prior to protein extraction. PCs were obtained from Centre of Drug Research (CDR, University of Helsinki, Finland) and diluted into DMSO (10 mM stocks).

Treatment compound	**Description**
DMSO (0.5 %)	Control (untreated)
5-Hydroxy-1,4-naphthoquinone (Juglone) (5 µM)	Control (Pin1 inhibitor)
Calyculin A (10 nM)	Control (PP2A inhibitor)
Allobarbital	Sedative
Butethal	Sedative
Clopenthixol dihydrochloride	Antipschotic
Selegiline hydrochloride	Monoamine oxidase inhibitor
Desalkylflurazepam	Hypnotic
Diazepam	Anxiolytic, muscle relaxant
Pentobarbital	Sedative

Blotting (semi-dry) of the proteins was performed using Bio-Rad Trans-Blot SD Semi-Dry Transfer Cell (20 V, 35 min). Pre-blotting soaked the extra thick blotting papers, pre-ran gels and PVFD-membranes (pre-soaked 1 min in methanol) for 10 min in 1× transfer buffer (1 × Semi-Dry Western blotting buffer; for 1 L; 100 ml 10 × Semi-Dry Western blotting buffer stock (480 mM Tris, 390 mM glycine, 13 mM SDS), 200 ml MeOH, 700 ml reverse osmosis H_2O). After blocking (5% milk powder in 1× TBST) and washing (1 × TBST) membranes were incubated with anti-bodies AT8 (Innogenetics, Invitogen) and TG3, which was a kind gift from Jin-Jing Pei (Karolinska Institutet, Stockholm, Sweden), PHF13 (Cell Signaling) and GAPDH (Millipore). For chemiluminescent detection, Pierce ECL Western Blotting Sub-

strate (Thermo scientific) was used and membranes were exposed on Kodak Biomax light films which were developed using a Kodak X-OMAT 1000 processor.

5.7 Phosphatase activity assay

The phosphatase activity assay was performed according to manufacturer's protocol, provided in the Serine/Threonine Phosphatase Assay System (V2460, Promega). First, cell lysates were prepared from 21 DIV RCNs (2 × 6-well plates, 400 000 cells/well). After the cells were washed once with ice-cold phosphatase storage buffer (PhSB, 50 mM Tris-HCl pH 7,4, 0,25 M sucrose, 0,1 M EDTA) cells were lysed in 75µl of ice-cold full PhSB (PhSB including 0,1% β-mercaptoethanol, protease inhibitor cocktail pill (complete Mini-EDTA free, Roche)) and collected using cell scrapers (modified from Planel et al. 2001). Cells from 6-wells were pooled into one sample tube, incubated on ice for 20 min and were pulled 10 times through a needle (1 ml syringe with 22 G × 1½″ needle). Pooled samples were centrifuged for 1 h at 100 000 × g at +4°C (Beckman TL-100 ultracentrifuge, TLA-55 rotor) and supernatants were then transferred into fresh tubes on ice. After stabilizing the spin columns, according to manufacturer's protocol, the cell lysates were pipetted into the spin columns, centrifuged for 5 min at 600 × g at +4°C to remove endogenous free phosphate and pooled the lysates resulting one lysate sample. Protein concentration of the cell lysate was determined.

The PP2A assay was performed according to manufacturers protocol (Phosphatase Assay protocol, Promega) using the supplied ½ -area flat-bottom 96-well plate in the final volume of 50 µl/reaction. The 5 × PPase buffer used was 5 × PP2A buffer (250 mM imidazole, 1 mM EGTA, 0,1% β-mercaptoethanol, 0,5 mg/ml bovine serum albumin (BSA)). All the reactions and proper controls were done in duplicates/triplicates and were incubated at 37°C for 30 min before stopping the reactions by adding the Molybdate dye/additive mixture. After 15 min of incubation in room temperature the PP2A activity was determined by measuring the absorbance (620 nm) of released phosphate-molybdate-malachite green complex.

5.8 Statistical analyses

Statistical analyses were performed using either the analysis of variance (three or more groups, which were followed by Bonferroni's post-tests) or using the Student's *t* test (two groups) in GraphPad Prism software. Significance was placed at $p < 0.05$.

6 RESULTS

6.1 Live-cell detection of tau interactions in PCA and HTS

6.1.1 Protein-fragment complementation assay

Tau protein is known to interact with numerous molecules in the cell environment under normal physiological and also under pathophysiologigal conditions. To study the interactions of tau in a living cell assay with its known interactors, multiple constructs were generated (figure 6). The luminescence signal of various tau interaction pairs was measured to confirm that the signal is detectable and feasible compared to control.

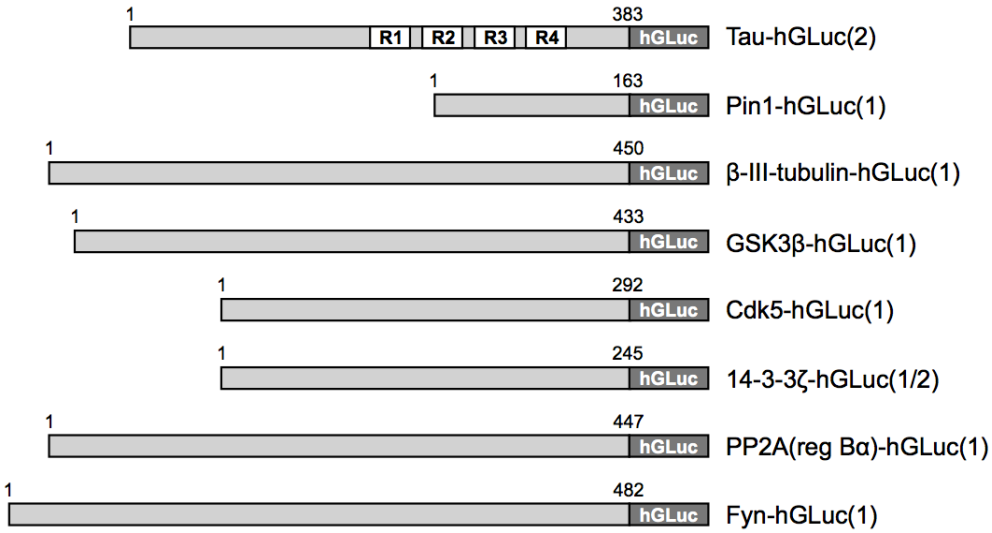


Figure 6. Schematic presentation of hGluc fragment-tagged reporter constructs. Schematic presentation of cloned constructs that were used to study protein-protein interactions of tau in live cells by using hGluc-based PCA strategy. In all constructs the hGluc tag was cloned in the C-terminus of the protein. Human tau isoform 0N4R was used throughout the study.

To test whether the hGluc-tag affects its cellular localization, phGluc(2C)-tau construct was overexpressed in cells with GFP-tubulin. Immunofluorescence microscopy experiments showed the localization of tau into neurites and cysoskeletal structures, suggesting that hGluc-tag does not interfere with the normal function and localization of tau in Neuro2A cells (figure 7A). Additionally to verification of hGluc-plasmids by DNA sequencing, the identity and expression of these fusion constructs were also verified in cells by Western blotting (figure 7B). Moreover, the responsiveness of PPIs to various inhibitors and other stimuli is crucial to confirm normal functionality. The major focus of the tau interacting molecules was protein kinases and protein phosphatases (PPs), which are known to contribute to the phosphorylation

equilibrium of tau, and molecules which participate in the regulation of phosphorylation state of tau and binding of tau into cellular structures (Pin1 and β-tubulin, respectively).

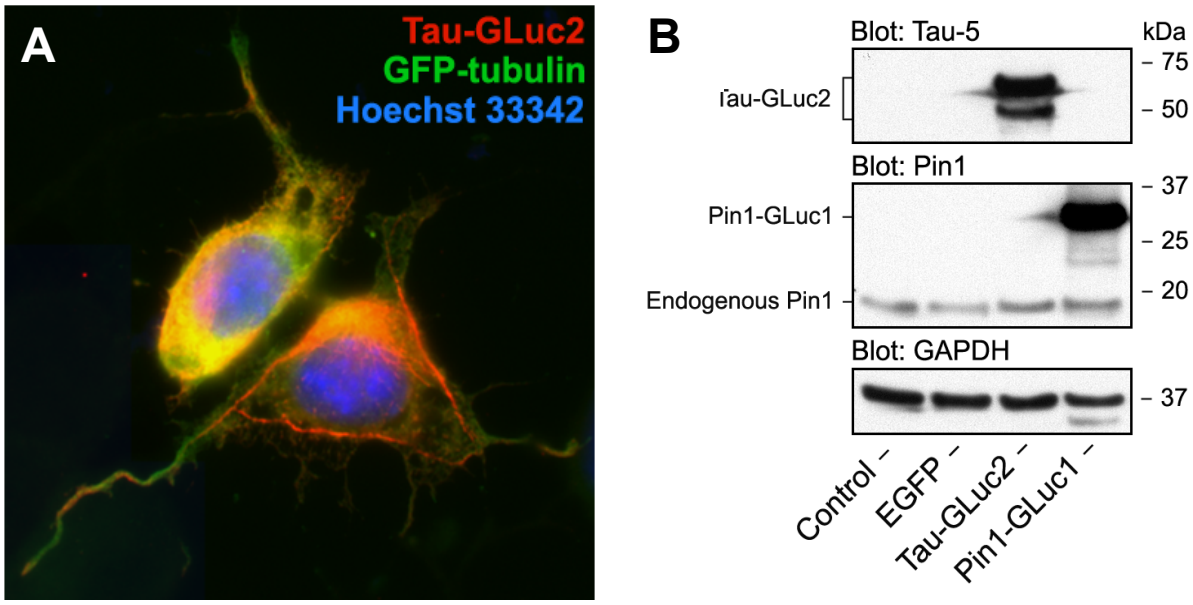


Figure 7. Subcellular localization of hGluc2-tau and the expression of the hGluc tagged fusion reporter proteins. Neuro2A cells were transiently transfected with hGluc2-tau and EGFP-tubulin to assess the normal localization of the tau fusion protein (A). The subcellular localization of hGluc2-tau (red) and EGFP-tubulin (green) were analyzed by immunofluerescence microscopy. Tau was detected with immunostaining using Tau-5 antibody. Localization of the fusion proteins to neurites and cytoskeletal structures in coexpressing cells indicates that hGluc fusion tag of tau does not interfere with its normal cellular functions and localization. The nuclei were counter-stained with Hoechst 33342. The expression of the hGluc tagged fusion reporter proteins was analyzed in Neuro2A cells by Western blot (B). The blots were stained with antibodies to tau (Tau-5) and Pin1. GAPDH antibody was used as a loading control.

6.1.2 PCA set up and optimization for High-throughput screening

The selection of the interaction pair for tau was the first step in the set up for PCA-based screening experiment. Based on the relatively high signal level and repetition of responsiveness to inhibition using juglone and to induction using KCl, Pin1 was chosen (figure 8A & B). Juglone, a known selective natural compound, is a cell-permeable and irreversible inhibitor of parvulin-like PPIases, such as *E. coli* parvulin and human Pin1 with IC50 of approximately 1.5 μM (Hennig et al. 1998). Furthermore, by increasing the media KCl concentration leading to cell depolarization has been reported to increase tau phosphorylation (Pierrot et al. 2006), which is also consistent with our PCA data. The critical facilitative function of Pin1 in dephosphorylation of specific disease-associated proline-directed serine and threonine sites on tau offers a feasible approach to study tau PPIs in this live-cell system.

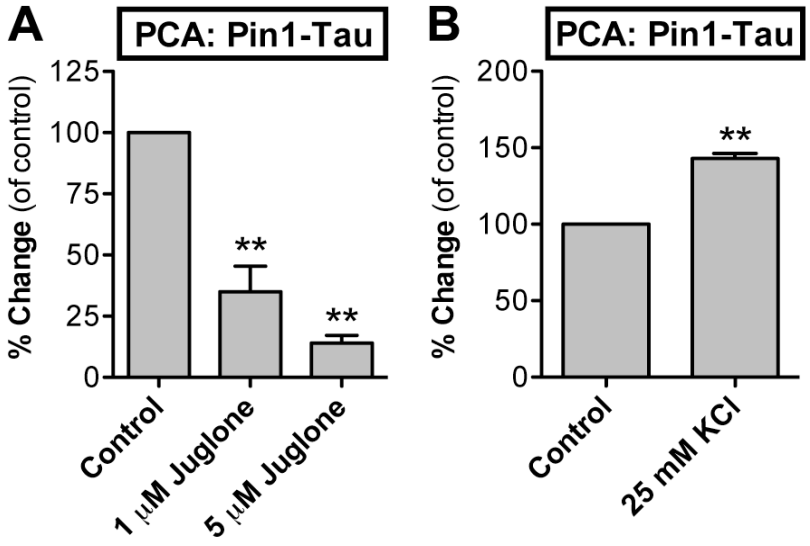
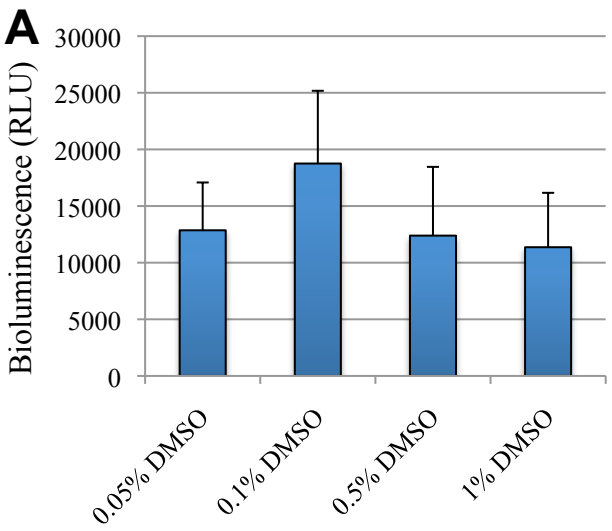


Figure 8. Validation of hGluc-based PCA for detection of Pin1-tau interaction. Pin1-tau interaction was pharmacologically validated to test the bidirectional response to stimuli. 48 h post-transfection, transiently transfected Neuro2A cells were treated with 1 μM or 5 μM Juglone (Pin1 inhibitor) (A) or 25 mM KCl (B). Luminescence signal was measured by flash luminometry in live cells followed by normalization of the values to corresponding data from β-galactosidase assay that was used as an internal vector control per well. The average values are displayed as percent change as compared to vehicle-treated control cells (means ± S.E.M.; 4 replicate wells per experiment, 4 independent experiments). ** indicate significant difference with $p < 0.01$.

To determine the optimal conditions for screening of small molecular libraries, different parameters were thoroughly studied. First, the maximum amount of vehicle (DMSO) that does not affect the basal signal and is not toxic to cells in our experimental set up time range was evaluated (figure 9A). The percentage of 0.5 % was determined for maximal concentration to be used in the screening. This decision was partly based on the concentration of the pre-diluted PC and NC master plates (10 mM and 20 mM, respectively). Second, the amount of cells per well in 96-well plate, ranging from 4 000-10 000, was tested using different transfection protocols with variable amount of DNA and transfection reagent (figure 9B). Using 10 000 cells per well resulted in the highest transfection efficiency and the lowest variation. Third, various measurement program protocols were tested using different measurement times and changing the pause time between the five measuments taken per well (figure 9C). Due to its low variability, although the signal level was slightly decreased, the protocol with 1 second measurement and a 0.2 second delay between the readings was chosen.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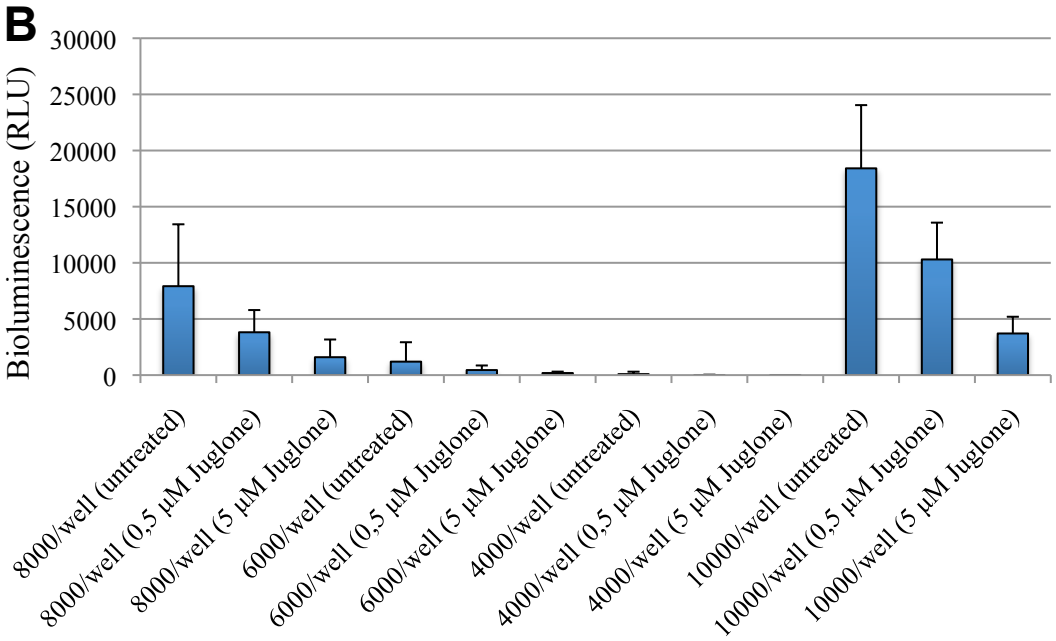
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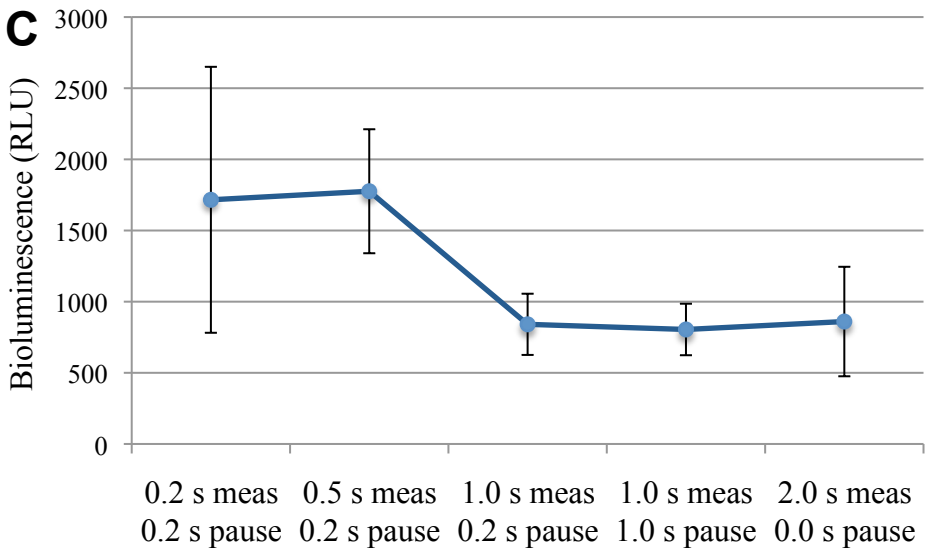
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Figure 9. Validation of optimal conditions of hGluc-based PCA for high-throughput screening. Various parameters were validated for optimal screening conditions of small molecular libraries. The maximum amount of vehicle (DMSO) that does not significantly affect the basal signal level and viability of the cells was tested (A). The number of cells per well ranging from 4000 to 10000 cells was tested (B). Also, depending on the cell number, different amounts of DNA and transfection reagent were used. For optimal measurement time frame window, five different measurement protocol programs were tested. Measurement times (m) and pauses between the measurements (p) varied from 0,2 seconds to 2 seconds and from 0,0 seconds to 1,0 seconds, respectively. Luminescence signal was measured by flash luminometry in live cells. RLU; relative luminescence units.

Bidirectional responsiveness of tau-Pin1 reporter system was further functionally studied. Co-overexpression of tau-Pin1 reporters with pcDNA3-GSK-3β resulted in a nearly two-fold increase in PCA signal compared to mock-transfected cells (empty pcDNA6-V5/His-C expression vector) (figure 10A). This effect was almost totally abolished in GSK-3β overexpressing cells by SB 216763, a specific GSK-3β inhibitor. Moreover, Neuro2A cells were transfected with either phGluc(2C)-tau alone or with pcDNA3-GSK-3β and treated with SB 216763, Calyculin A or vehicle. After the protein extraction, the level of tau phosphorylation at specific epitopes was detected on Western blots. The data from the immunoblots showed that the overexpression of GSK-3β increases the phosphorylation of tau at least in epitopes AT8 and PHF13 (figure 10B). Altogether, these functional validation data suggest that tau-Pin1 interaction pair responds to both biochemical and genetical stimuli in a predictable way providing evidence of feasibility to further investigate this interaction.

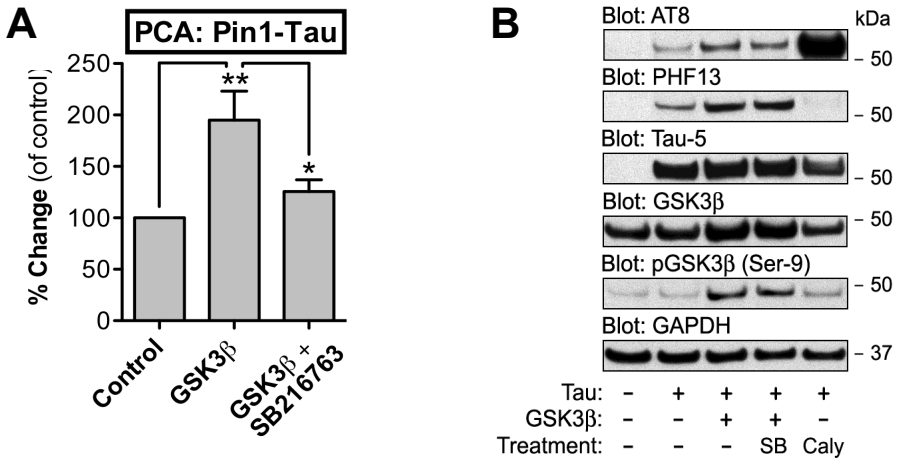

Figure 10. Validation of hGluc-based PCA for detection of Pin1-tau interaction by using GSK-3β overexpression. Pin1-tau interaction was genetically validated by co-overexpression of GSK-3β to test its effect on PCA signal and tau phosphorylation status. Neuro2A cells were cotransfected with reporter constructs hGluc1-Pin1 and hGluc2-tau together with either empty mock plasmid (control) or with pcDNA3-GSK-3β (A). The vehicle treated cells co-transfected with pcDNA3-GSK-3β generated nearly two fold signal compared to control cells

and to pcDNA3-GSK-3β cotransfected cells treated with GSK-3β inhibitor SB 216763. Luminescence signal was measured by flash luminometry in live cells followed by normalization of the values to corresponding data from β-galactosidase assay that was used as an internal vector control per well. The average values are displayed as percent change as compared to vehicle-treated control cells (means ± S.E.M.; 4 replicate wells per experiment, 4 independent experiments). GSK-3β overexpressing cells were analyzed by Western blot (B). Neuro2A cells were transiently transfected with empty mock plasmid or hGluc2-tau with or without pcDNA3-GSK-3β as indicated. Before harvest, the cells were treated for 2 h with vehicle, 100 nM SB 216763 or 10 nM Calyculin A. The blots were stained with phospho-specific tau antibodies PHF13 and AT8 and Tau-5 to detect total tau levels. To verify GSK-3β overexpression, the blots were stained with GSK-3β and phospho-GSK-3β (Ser9) antibodies. GAPDH antibody was used as a loading control. * and ** indicate significant differences with $p < 0.05$ and $p < 0.01$ respectively. SB; SB 216763, Caly; Calyculin A.

6.1.3 High-throughput screening

Based on the validation data, the screening of small molecular compounds using tau-Pin1 interaction PCA was performed. First, the treshfold percentage to identify possible hits was determined for increased PCA signal (200%) and decreased PCA signal (75%) compared to DMSO vehicle control. The interaction was screened with focused libraries of known pharmaceutical compounds (240) and natural compounds (115) (appendices 1 and 2, respectively). There were two drug classes that most commonly repeated among the hit compounds. Sulfonamide antibacterials and sedative-hypnotics comprised more than 40% of the hits in tau-Pin1 screen (table 6). Noticeable differences were detected in the PCA signals between the wells treated with these small molecular modulators of the libraries, which implicates that the assay is responding to stimuli, and, thereby suggests that the PCA approach is suitable for screening small molecular modulators of tau PPIs (figure 11).

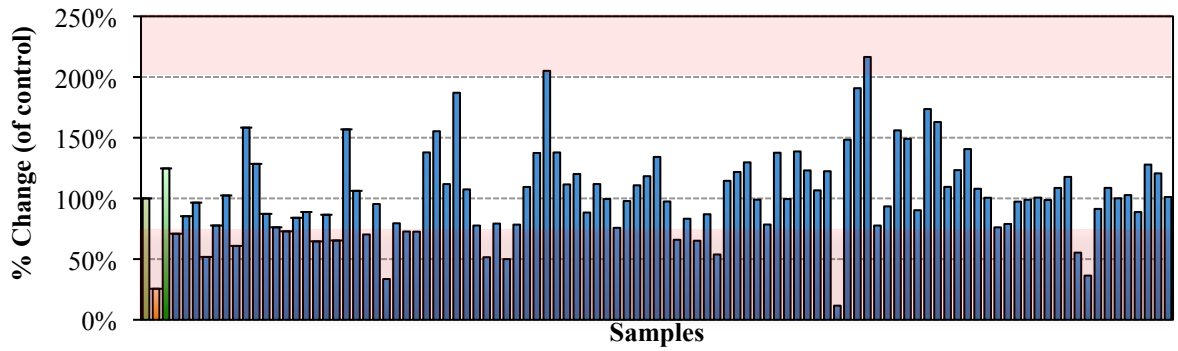

Figure 11. An example of raw data obtained from five combined 96-well plates from primary screening of small molecular modulators of Pin1-tau interaction. Each bar (blue) represents the luminescence values detected from Pin1-tau PCA from four replicate wells per compound. The average values are displayed as percent change as compared to vehicle-treated control (dark green) cells. 5 μM juglone (orange) and 25 mM KCl (light green) were used as controls

on each 96-well plate. Hit area (above 200% or below 75%) is marked in semi-transparent red color.

The primary screen yielded 26 preliminary hits from the pharmaceutical compound library from which 21 increased and 5 decreased tau-Pin1 PCA signal (table 6). 2 out of 5 initial hits that decreased the PCA signal were determined as false positive hits (appendix 3, false positive hits not included). This was due to incompatibility of these compounds in luminescence-based PCA screen resulting in almost total loss of signal of tau-Pin1. Furthermore, these incompatible compounds were verified as false positive hits according to identical results obtained from the screen performed simultaneously on APP metabolism by Prasanna Sakha (Neuroscience Center, University of Helsinki, Helsinki, Finland) (unpublished data). The screen from the natural compounds also yielded a few hits, from which all decreased the signal. Although among these hits there were some very interesting compounds included, the rest of the study was decided to proceed through by only investigating the hits from the pharmaceutical compounds and leave the natural compounds for further studies.

Table 6. Summary of hits of pharmaceutical compounds from tau-Pin1 screening.

Compounds screened	240
Total number of hits	26
Total number of excluded hits based on assay incompatibility	2
Hit rate	10.0%
Total number of compounds increasing the interaction	21
Total number of compounds decreasing the interaction	3
Most commonly repeated drug classes:	
- Sedatives (barbiturates or benzodiazepines):	5
- Sulfonamide antibacterials:	3

Next, we confirmed the hits in dose-response assays. 14 compounds were chosen for secondary screening (appendix 4). These 14 compounds were selected based on the signal levels from the first screen and their compound characteristics, which included predetermined therapeutic functions and ruling out compounds that would be incompatible for CNS use (e.g. topically applied antiseptics). Moreover, in the dose-response experiments the effect of sulfonamide antibacterials could not be consistently confirmed. In contrast, the secondary screening verified the increase of the tau-Pin1 interaction observed in the primary screen by sedative-hypnotics. The increase of the sedative treated tau-Pin1 signal compared to vehicle treated cells varied between 1,4-2,0-fold depending on the compound used. However, the increase in tau-Pin1 PCA signal was slightly lower in the secondary testing compared to primary screen.

The effect of two sedative-hypnotics and folic acid, which was one of the few compounds identified to decrease tau-Pin1 interaction, were further validated in PCA using internal vector control (figure 12). Butethal, a barbiturate, and desalkylflurazepam, a benzodiazepine, both dose-dependently increased tau-Pin1 interaction, whereas consistent dose-dependent decrease was observed in cells exposed to folic acid.

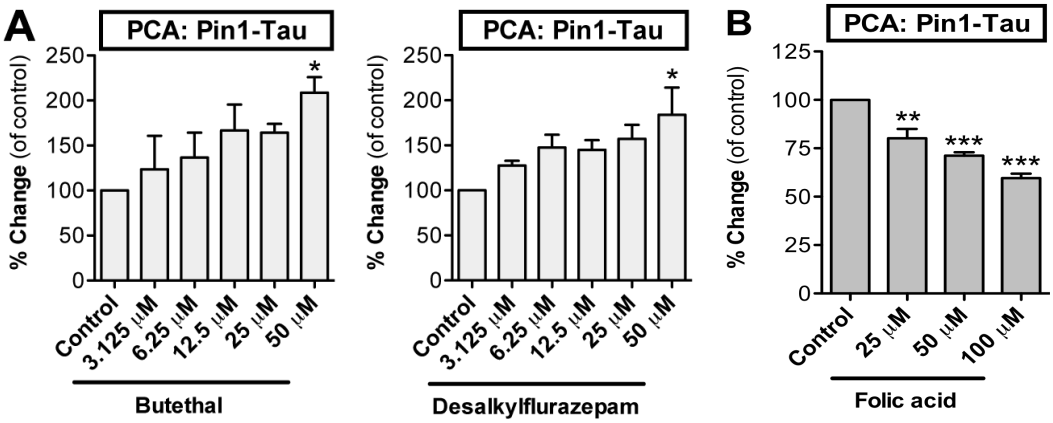

Figure 12. Validation of hit compounds with hGluc-based protein-fragment complementation assay (PCA) for detection of tau-Pin1 interaction. Transiently transfected (constructs hGluc1-Pin1 and hGluc2-tau) Neuro2A cells were treated for 2 h with increasing consentrations of butethal (A, left panel) or desalkylflurazepam (A, right panel) or folic acid (B) 48 h post-transfection. Luminescence signal was measured by flash luminometry in live cells followed by normalization of the values to corresponding data from β-galactosidase assay that was used as an internal vector control per well. The average values are displayed as percentages of change as compared with vehicle-treated control cells (means ± S.E.M.; *n* = 3). *, **, and *** indicate significant differences with $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

6.2 Pharmacological modulation of neurons and mechanistic studies

6.2.1 Treatments of mature RCNs and Western blot analyzes

The sedative-elicited increase in tau-Pin1 PCA signal that was detected in the screening was subsequently examined in mature (21 DIV) rat cortical neurons (RCNs). To test whether the increased signal was due to increased phosphorylation of tau, RCNs were treated with 7 different compounds for 2 h with proper controls included (table 5). From the compounds tested on mature RCNs, butethal and desalkylflurazepam showed significant increases in tau phosphorylation at AT8 phosphoepitope (Ser199/Ser202/Thr205) analyzed on Western blot (figure 13). Unexpectedly, large differences were seen in tau phosphorylation status between the different compounds within the same compound classes (barbiturates and benzodiazepines).

Hence, according these data, barbiturate butethal and benzodiazepine desalkylflurazepam were chosen for further studies.

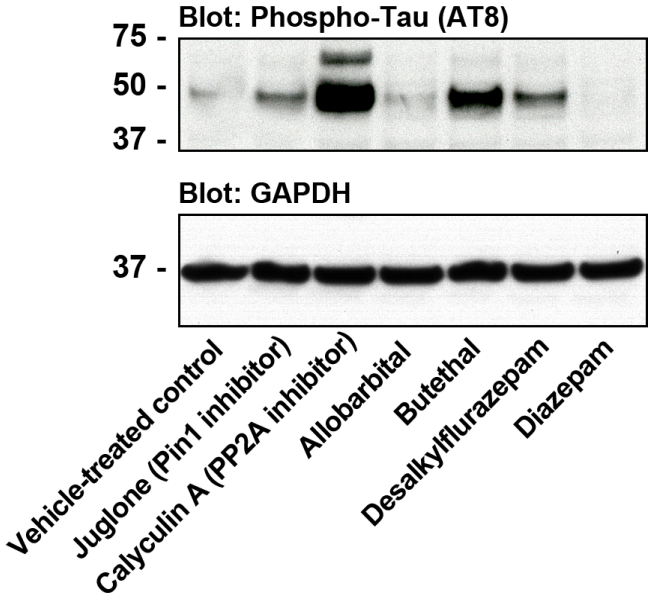

Figure 13. Effect of hit compounds on mature rat cortical neurons. 21 DIV rat cortical neurons (RCNs) were treated for 2 h with various hit compounds identified from the screen of small molecular modulators at 50 μM concentration. Different compounds (4 out of 7 tested depicted here) showed large variation in the phosphorylation status of tau at AT8 phosphoepitope even within the same compound classes (barbiturates; allobarbital and butethal, benzodiazepines; desalkylflurazepam and diazepam) when analyzed by Western blot. Juglone and Calyculin A was used as controls. GAPDH antibody was used as a loading control.

Next, dose-dependent experiments were performed using these two sedatives with lower concentrations (0,1 μM, 1 μM and 10 μM) compared to previous treatments (50 μM). Additionally, the treatment time was also extended to 6 h with the exception of controls, which remained in 2 h because of their potential long-term toxicity to neurons. Besides AT8 phosphoepitope, other AD-related tau epitopes were examined on Western blot. Treatments with increasing doses of butethal and desalkylflurazepam, which both are positive modulators of γ-aminobutyric acid type A ($GABA_A$) receptors, resulted in increased tau phosphorylation at AT8 epitope, whereas no alterations in phosphorylation status were detected when probed with TG3 (Thr231/Ser235) or PHF13 (Ser396) phosphoepitopes (figure 14A & B).

Furthermore, to study the stability of the effect observed with these compounds, a washout experiment was performed. RCNs were treated with 50 μM butethal or desalkylflurazepam for either 24 h or 48 h. 24 h treatments were followed by a washout period of 24 h (i.e. after the incubation of 24 h with compound, the culture media was replaced to fresh full NB without the compounds for a period of 24 h before the protein extraction). The phosphorylation at

AT8 epitope remained at elevated level after the 24 h washout period compared to vehicle treated neurons (figure 14C). Taken together, butethal and desalkylflurazepam both increase tau phosphorylation at the AT8 epitope and the effect is clearly detectable even after the washout period of 24 h.

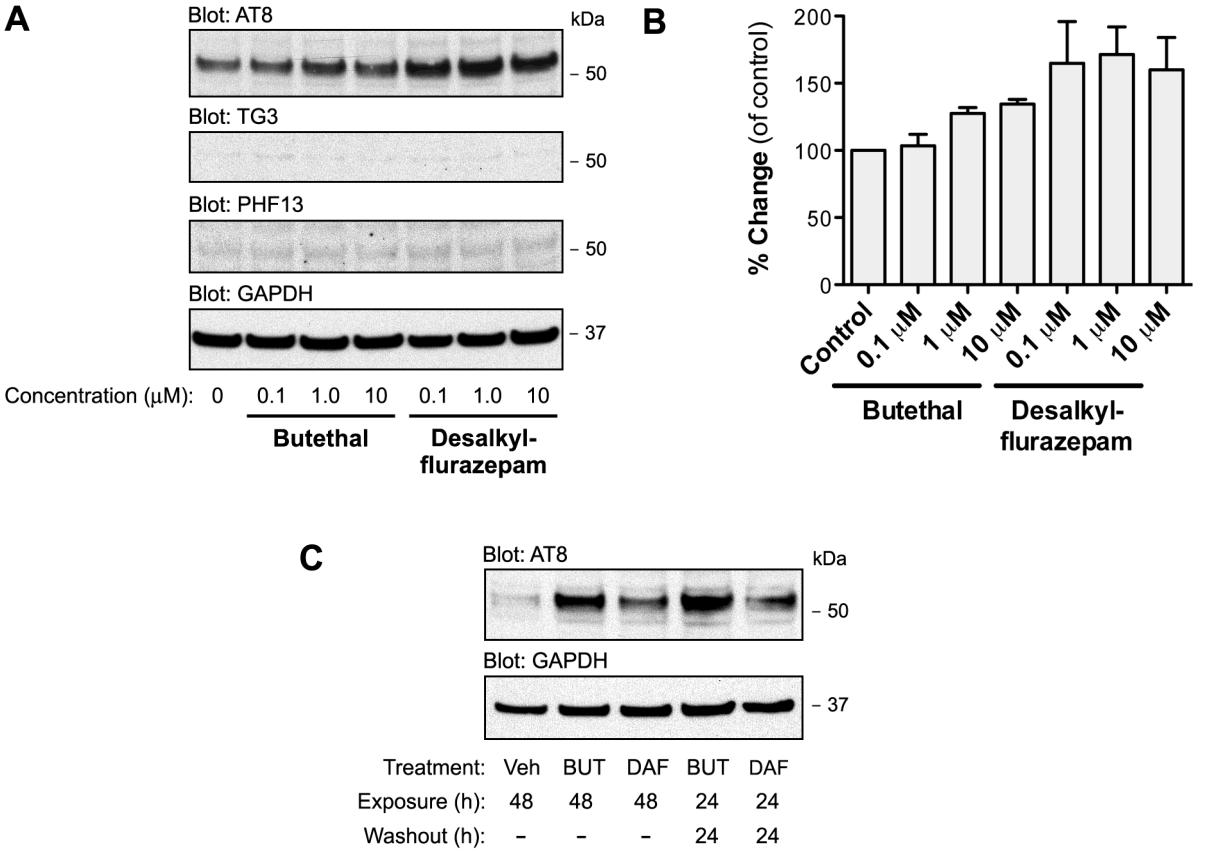

Figure 14. Effect of $GABA_A$ receptor modulators on mature rat cortical neurons. Mature primary rat cortical neurons (21DIV) were treated with three different concentrations of butethal and desalkylflurazepam for 6 h (A). Western blot analyzes were performed to cell extracts using phosphospecific tau antibodies (AT8, TG3 and PHF13). Treatments with both butethal and desalkylflurazepam increased the phosphorylation of tau at AT8 epitope compared to vehicle treated controls. (B) Optical density quantification of the AT8 epitope phosphorylation of tau from Western blot (figure 14A). (C) 21 DIV primary rat cortical neurons were treated 50 μM butethal and desalkylflurazepam for 48 h or treated for 24 h followed by a washout period of 24 h where drugs were removed from the cells and replaced with fresh media without the $GABA_A$ active drugs. As compared to vehicle treated controls, increased phosphorylation of tau at AT8 epitope induced by butethal and desalkylflurazepam remained at an elevated level after the 24 h washout period. GAPDH antibody was used as a loading control. BUT; butethal, DAF; desalkylflurazepam, Veh; vehicle.

6.2.2 Role of PP2A in sedative induced increase in tau phosphorylation

PP2A is the major protein phosphatase suggested to dephosphorylate tau and its interaction with tau is dependent on Pin1-catalyzed *cis*/*trans* proline isomerization that preceeds the

phosphorylated serine/threonine residues. The PP2A activity was studied from 21 DIV rat cortical neurons to assess if the sedatives have a direct effect on PP2A activity *in vitro*. In this cell-free assay, either butethal or desalkylflurazepam does not seem to directly decrease PP2A activity while calyculin A, a PP2A inhibitor, has a significant effect reducing the enzyme activity (figure 15A).

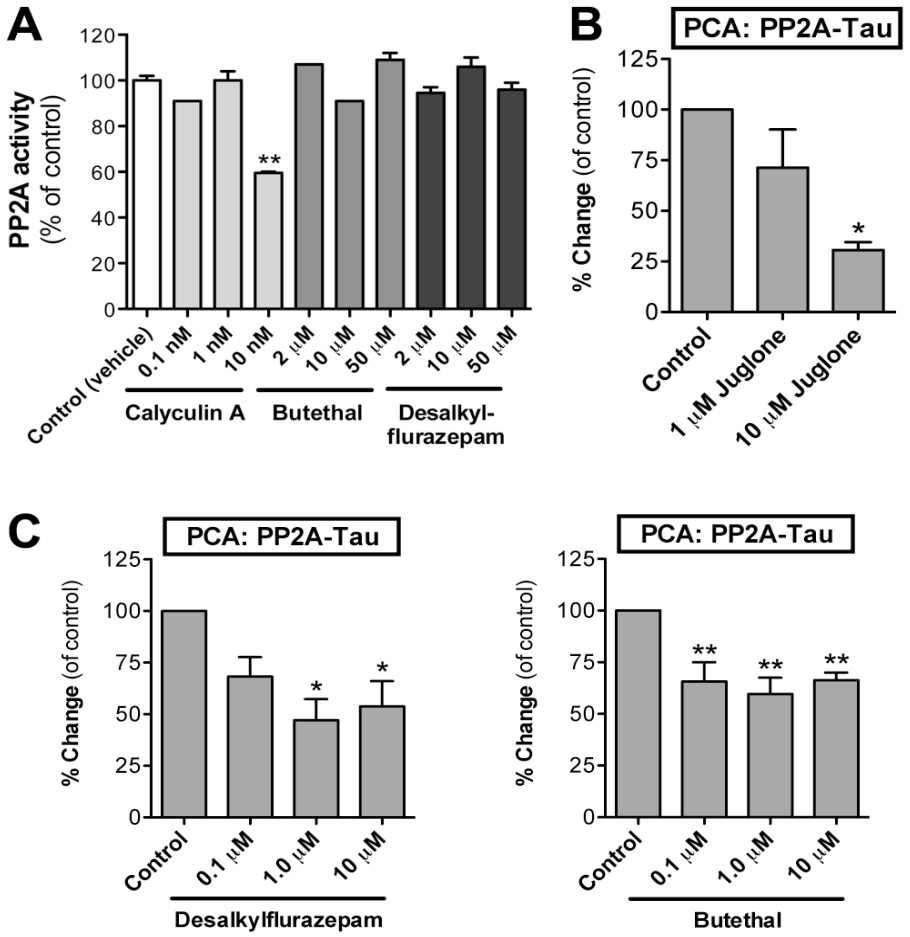

Figure 15. Enzymatic activity of protein phosphatase 2A (PP2A) in a cell-free assay and PP2A-tau interaction in Neuro2A cells. To test the direct effects of $GABA_A$ receptor activators on PP2A activity, the samples were treated with butethal and desalkylflurazepam in a cell-free assay (A). The enzymatic activity of PP2A was not altered when treated with $GABA_A$ active drugs, whereas Calyculin A treatment, a potent PP2A inhibitor, resulted in significant reduction in the enzymatic activity. However, decrease of nearly 50 % in PP2A-tau interaction was detected in intact transiently transfected cells when treated with $GABA_A$ receptor modulators (C). Juglone (Pin1 inhibitor) was utilized in the pharmacological validation of the PP2A-tau interaction (B). The average values are displayed as percentages of change as compared with vehicle-treated control cells (means ± S.E.M.; $n = 3$). * and ** indicate significant differences with $p < 0.05$ and $p < 0.01$, respectively.

The interaction of tau and PP2A was also studied using PCA platform. Since PP2A-tau interaction depends on Pin1 activity, PP2A-tau PCA was validated with Juglone (1 μM and 10

μM) to test its functionality and the effect of the sedatives was further studied (figure 15B). Treatment of Neuro2A cells overexpressing tau and PP2A hGluc plasmids with these sedatives showed a reduction of nearly 50% in the PCA signal (figure 15C). Although butethal or desalkylflurazepam do not affect the enzyme activity of PP2A in cell-free *in vitro* assay, the interaction of these proteins in intact cells is clearly reduced.

The results presented in this MSc. thesis have been published in The Journal of Biological Chemistry (Vol. 287, No. 9, pp. 6743–6752, February 24, 2012) by Niko-Petteri Nykänen, Kai Kysenius, Prasanna Sakha, Päivi Tammela and Henri J. Huttunen. All of the experimental procedures and the results presented in here were performed and produced by the author of this thesis unless otherwise mentioned.

7 DISCUSSION

7.1 Experimental procedures and results

In the cellular environment, there is a constant flux of a myriad of molecular interactions, which are highly sensitive to alterations in the ambient conditions. When using live cell approaches, it is highly important to try to minimize the fluctuation in the data by using exactly the same conditions throughout a single experiment and in the subsequent replicate experiments. Factors that may create changes in the normal physiological state of the cell, such as transfection to induce overexpression of certain proteins, treatment with modulators of specific cellular functions and exposure times should be under careful consideration. Thus, thorough functional validation of each experiment, and in this case each PPI pair separately, is instrumental when working with live cell assays. Furthermore, the selection of the cell line or primary cells used is essential to obtain information from the target of interest and also to assess which cells are the most feasible in a certain phase of an experiment. Moreover, the characteristics of the cells used, such as endogenous expression of specific proteins and suitability of the cells (e.g. transfection efficiency, division rate) to experimental workflow are crucial properties to produce reliable and repeatable data. For example, mouse neuroblastoma 2A cells, which were mostly employed in this study, are neural like tumor-derived cells with a rapid division cycle and high transfection efficiency properties, which provides feasible basis to study protein-protein interactions. Nevertheless, the suitable properties of Neuro-2A to investigate PPIs, the networking of the cells and structural and functional properties still highly differs from primary neurons, which emphasizes the further utilization of neurons as a proper

in vitro study platform to investigate neuronal phenomena. However, primary neurons pose other technical challenges, such as difficulty to transfect with high efficiency.

Reliable and repeatable positive and negative controls are fundamental for interpretation of the data regardless of the experiment design. To rule out any false positive signal in PCA experiments, only one of the split luciferase fragments including the insert of protein of interest was transfected. These cells did not emit any detectable luminescence compared to untransfected cells and therefore were used as a background control (data not shown). To further rule out the additional false positive results, the spontaneous folding of the complementary luciferase enzyme fragments without any fusion proteins were studied providing similar results as the transfection of only other hGluc fragment. Moreover, the tagging of the protein of interest with e.g. His-tag, TA-tag or hGluc-tag may effect on the physiological function and the subcellular localization of the protein. In this study it was shown that hGluc-tagged tau colocalizes with GFP-tubulin to cytoskeletal structures and neurites suggesting that the hGluc-tag does not interfere the normal function and cellular localization of tau. Moreover, hGluc-tagged tau was normally phosphorylated in multiple epitopes in response to GSK-3β overexpression.

Because of the critical role of Pin1 in tau dephosphorylation in neurons (Lu et al. 1999) and the encouraging results obtained from the primary PCA experiments, Pin1-tau interaction was chosen as a dynamic live cell protein-protein interaction reporter to focus on further studies. The data from the functional validation experiments showed that the bidirectional responsiveness of Pin1-tau PCA to genetic stimulation using GSK-3β overexpression, and also to pharmacological modulation using Juglone for inhibition of Pin1. Western blot studies of Neuro-2A cells, which were transfected with either tau-Gluc fusion protein alone or cotransfected with GSK-3β showed that GSK-3β overexpression promotes phosphorylation of tau fusion protein at probed AT8 and PHF13 epitopes. Hence, based on these data, it was concluded that hGluc-based protein-fragment complementation assay is suitable for studying tau PPIs in live cells.

After the functional validation of the PCA assay, a high-throughput screening (HTS) was performed to search for molecules to increase or decrease tau-Pin1 interaction. In this case, a proof-of-concept screen is a more adequate term to describe the screening than HTS because of its strong focus to known pharmaceuticals and natural compounds and, also, the rather limited number of molecules screened compared to general description of HTS, in which the libraries may contain small molecular compounds from 100 000 to even up to one million. Nevertheless, the proof-of-concept screening showed that the PCA based assay is capable of

detecting novel modulators of tau PPIs. For example, folic acid, which was one of the few compounds identified from the screen to decrease the Pin1-tau interaction, has been previously reported to affect the methylation and maturation of PP2A complex thereby resulting in altered tau phosphorylation (Sontag et al. 2008). This is consistent with the data presented here showing that folic acid is reducing the tau-Pin1 interaction signal levels and, therefore, likely correspond with the phosphorylation state of tau. Interestingly, it has been reported that higher folate intake is associated with reduced incidence of Alzheimer's disease in the elderly (Luchsinger et al. 2007).

The results of the proof-of-concept screen identified two compound classes that increased the tau-Pin1 interaction. These somewhat unexpected hit compounds belonging to the barbiturates and benzodiazepines are both known modulators of γ-aminobutyric acid type A ($GABA_A$) receptors (e.g. Jacob et al. 2008). Neuro-2A cells, which were the cells utilized in the proof-of-concept screen are known to express functional $GABA_A$ receptors (Baraldi et al. 1979), which suggests that the observed increase in Pin1-tau interaction may result from enhanced GABA signaling. Previously it has been shown that tau is functionally linked to trafficking of NMDA receptors (Ittner et al. 2010) and there are also implications of increased tau phosphorylation in response to dopamine receptor D1 activation (Lebel et al. 2009; Lebel & Cyr 2011). To date, with the exception of those previously mentioned examples, tau has not been directly functionally linked in other neurotransmitter receptor systems. On the other hand, interesting findings have been observed in the modulation of tau phosphorylation in the cortex and hippocampus induced by various anesthetics that also affect $GABA_A$ transmission in the brain (Planel et al. 2007). Moreover, it has been suggested that PHF-like hyperphosphorylation of tau is a reversible event in hibernating mammals (Arendt et al. 2003). In addition to different neurotransmitter systems and central nervous system (CNS) active drugs responsible for altered tau phosphorylation, this may implicate a more general association between the level of neuronal activity and tau phosphorylation.

The most of the fast inhibitory neurotransmission in the mammalian brain is mediated by signalling through $GABA_A$ receptors ($GABA_ARs$) (e.g. Jacob et al. 2008; Luscher et al. 2011). The $GABA_ARs$ belong to the superfamily of ligand-gated ion-channels and are abundantly expressed in neurons throughout the CNS and, also, are clinically significant drug targets for multiple CNS-active agents such as anti-convulsants, sedative-hypnotics and anxiolytics. $GABA_ARs$ comprise a family of ligand-gated chloride channels wih a vast number of various subunit composition. 19 different $GABA_AR$ subunits have been identified to date, which are divided into eight subunit classes (α1-6, β1-3, γ1-3, δ, ε, θ, π, ρ1-3) based on their sequence

homology (e.g. Luscher et al. 2011). In addition, the alternative splicing of receptor mRNAs further increases the structural diversity of $GABA_A$Rs. However, the most common receptor subunit composition includes 2α and 2β subunits with an additional single γ or δ subunit. Furthermore, $GABA_A$Rs formed by pentameric assembly of multiple different subunits can be targeted to distinct subcellular regions and the expression levels of these $GABA_A$Rs may vary largely due to a receptor subunit composition differences in specific cellular locations (e.g. Jacob et al. 2008; Luscher et al. 2011). Also, differently formed $GABA_A$Rs possess distinct pharmacological and physiological properties. For example, a composition containing α1, α2, α3 or α5 subunits together with β and γ subunits are benzodiazepine-sensitive, mainly synaptically located and mediate the most of the phasic inhibition in the brain (Brunig et al. 2002). By contrast, $GABA_A$Rs composed of α4 or α6 subunits associated with β and δ subunits are insensitive to benzodiazepine modulation. These receptors mediate tonic inhibition in the brain and form a distinct population of extrasynaptic (i.e. $GABA_A$Rs that are not located at synaptic sites) receptors. The data from the experiments performed with mature sypatically connected cortical neurons suggests that the effect of benzodiazepine-induced increase in tau phosphorylation is for the most part mediated by synaptic $GABA_A$ receptors.

Benzodiazepines (BZ) are commonly used hypnotics, anti-convulsants and anxiolytics that are routinely utilized in presurgical anesthesia due to their potent sedative properties. Development of tolerance and addiction are both known adverse effects of BZs, which is the reason why long-term usage of BZs has not been approved. BZs and ethanol, which are both associated with anterograde amnesia, shares several pharmacological actions and they both act mostly or partially through $GABA_A$ receptors (Curran 1991; Fadda & Rossetti 1998). Furthermore, increased tau phosphorylation at PHF-1 epitope and Ser199 has been reported in developing neurons induced by ethanol exposure (Saito et al. 2010). The data from the experiments in cultural neurons suggests that tau phosphorylation at the AT8 epitope is a downstream effect induced by $GABA_A$R activation and, this phosphorylation further remains at elevated level for at least 24 h after the washout of $GABA_A$R activating drugs. These observations raise intriguing questions of the mechanistic associations between the chronic use of sedatives or ethanol, anterograde amnesia, and possibly even increased risk of dementia.

Protein phosphatase 2A (PP2A) has been shown to associate with and dephosphorylate $GABA_A$R subunit β3, a subunit which phosphorylation appears to play an important role in the regulation of $GABA_A$R function (Jovanovic et al. 2004). The data presented here suggests that activation of $GABA_A$Rs enhances tau phosphorylation and increases tau-Pin1 interaction

but decreases the association between PP2A and tau. Furthermore, based on the PP2A phosphotase activity assay, the enzyme activity of PP2A is not inhibited directly by $GABA_AR$ modulators *in vitro*. However, the tau-PP2A PCA data showed a decrease in the interaction when treated with $GABA_AR$-active sedatives, which may at least partly result from the compartment-specific differences in PP2A expression and enzyme activity due to the polarized structure of neuronal cells. Hence, the recruitment of PP2A for $GABA_AR$ β3 subunit dephosphorylation and receptor desensitization that is possibly induced by strong and persistent $GABA_A$ receptor activation, could reduce the PP2A availability to dephosphorylate tau. Moreover, the association of synaptic activity, PP2A and their contribution to tau phosphorylation has been further studied. It has been suggested that synaptically released zinc could play an important role in tau hyperphosphorylation (Sun et al. 2012). It was shown that in cultured hippocampal slices and primary neurons, the blockade of zinc release in the synaptic terminals highly decreased the tau hyperphosphorylation associated to synaptic activity and, also, that synaptic activity-induced hyperphosphorylation was strongly associated to PP2A inactivation. The suggestion that increased phosphorylation of tau is induced by synaptically released zinc through inhibition of PP2A, which was actually reported to be reversed by zinc chelators, provides another implication between synaptic activity and deregulation of tau phosphorylation. However, whether the synaptic activity contributes to deregulation of tau phosphorylation or is a merely another physiological tau function remains to be clarified by future studies.

7.2 Conclusions and future experiments

Regulation of tau phosphorylation and dephosphorylation is a highly controlled event in neurons. Disturbed equilibrium of this dynamic system may contribute to development of various tauopathies, such as Alzheimer's disease and frontotemperal dementia. Therefore, more detailed understanding of the highly complex dynamics of protein-protein interactions of tau is required to respond to the emerging need to develop novel tau-targeted therapies for aging-related CNS disorders. However, different approaches have been used in order to identify compounds or molecules that could potentially modulate tau phosphorylation from a disease-modifying aspect regarding to neurodegenerative diseases (e.g. Ballatore et al. 2007).

In conclusion, based on the data presented here, PCA-based novel dynamic assay is a method capable for measuring protein-protein interactions of tau in live cells. Validation of the assay showed that Pin1-tau interaction is responding to both pharmacological and genetic

modulation thereby confirming its feasibility and reliability to further investigate tau interactions and search for possible novel molecules and compounds to decrease or increase specific tau PPIs. After the parameter validations, the proof-of-concept screening was performed using the focused libraries of pharmaceutical and natural compounds. The hits from the screen identified a class of physiologically relevant compounds belonging to $GABA_A$ active drugs. The effect of these benzodiazepines and barbiturates were further studied on mature synaptically connected cortical neurons confirming an increase in tau phosphorylation. Moreover, desalkylflurazepam and butethel induced the phosphorylation of tau at the AT8 epitope (Ser199/Ser202/Thr205) as a downstream effect of $GABA_A$ receptor activation and the phosphorylation remained at an elevated level for at least 24 h after the washout of the drugs. Interestingly, the tau-PP2A interaction was decreased when treated with desalkylflurazepam and butethal. However, this effect is not directly mediated by the inhibition of PP2A enzyme activity in a cell free assay.

The exact mechanism how $GABA_A$ receptor activation is associated to the observed increase in tau phosphorylation is yet to be established. The major inhibitory synapse scaffolding protein gephyrin that is directly linked to microtubules associates with $GABA_AR$ complexes (Prior et al. 1992; Essrich et al. 1998). As a highly abundant MT-binding protein, tau could also play a role in a dynamic process of clustering regulation or contribute to trafficking of $GABA_ARs$ at the inhibitory synaptic sites. Moreover, tau may consequently become a subject of modulation by GABAergic synaptic signaling, which could be manifested as an aberrant phosphorylation. Furthermore, the investigation if tau and gephyrin are directly or indirectly connected could bring novel insights about the $GABA_AR$ clustering and trafficking.

The regulation of tau phosphorylation *per se* is a highly complex system, which is affected by multiple protein kinases and phosphatases. Although GSK-3β and CDK5 have been referred as major kinases in tau phosphorylation, more detailed understanding is required about the contribution of other kinases and their priming kinase effects on phosphorylation, and, also on the temporal sequence of phosphorylation and how it is affected by the primed phosphosites. In addition to phosphorylation, numerous other post-translational modifications, such as glycosylation and acetylation, regulate tau function. Hence, the overall impact of the numerous possible tau modifications needs more detailed investigation in order to establish the potential tau species that contribute to neurodegeneration of multiple tauopathies.

A growing number of reports from e.g. amyloid-β peptide targeted clinical trial discontunuations sets more and more emphasis to alternative approaches to treat and diagnose AD and other related forms of dementia. An emerging field of identifying biomarkers for specific

diseases is essential for earlier diagnosis of neurodegenerative disorders (ND). Because the asymptomatic stage of certain NDs can last for years or even decades before the clinical manifestation, the early diagnosis, or at least exclusion of related disorder, is instrumental to search for novel symptomatic, neuroprotective or even disease-modifying therapeutics. Interestingly, besides the conventional role of tau as a cytoskeletal stabilizer protein, novel functions of tau have been described. For example, it has been suggested that hyperphosphorylation-induced dendritic (mis)localization of tau may directly promote synaptic dysfunction in the dendritic spines (Hoover et al. 2010) and induce synaptotoxicity of β-amyloid via the NMDA receptor which is associated to Fyn transport in dendritic postsynapse (Ittner et al. 2010). These intriguing novel aspects of tau function and dysfunction further substantiate the importance to study tau-driven neurodegeneration in tauopathies for more extensive understanding and targeted therapeutics of these disorders. Importantly, idiopathic neurodegenerative disorders are not generally manifestations of a single disease-specific predominant protein but combinations of concurrent and concomitant pathophysiological processes that eventually lead to neurodegeneration and subsequent disease-specific symptoms.

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APPENDICES

Appendix 1. List of screened pharmaceutical compounds.

Pharmaceutical compounds (PC)

Code	Name
PC001	Acetaminophen, Paracetamol
PC002	Acetanilide
PC003	Acetazolamide
PC004	Acetyleneurea, Acetylene carbamide
PC005	N-Acetylsulfanilamide
PC006	Acetylsulfisoxazole
PC007	L-3-(3,4-Dihydroxyphenyl)alanine, L-DOPA
PC008	Allobarbital
PC009	Allylestrenol, 17-Alpha-allylestr-4-en-17-beta-oll
PC010	p-Aminobenzoic acid
PC011	4-Aminoantipyrine, Ampyrone
PC012	Amydricaine hydrochloride
PC013	4-Alpha-Androstane
PC014	Antazoline, hydrochloride
PC015	Antipyrine
PC016	Arecoline hydrobromide
PC017	Atenolol
PC018	Atropine methyl nitrate
PC019	Bamethan
PC020	Barbital
PC021	Barbituric acid
PC022	Benzalkonium chloride
PC023	Benzocaine
PC024	Bromhexine hydrochloride
PC025	Buclosamide
PC026	Bunitrolol
PC027	I-Bunolol
PC028	Busulfan
PC029	Butethal
PC030	Butidrine
PC031	Butyl 4-Aminobenzoate
PC032	p-Butylaminobenzoyldimethylaminoethanol, Cainopan, Tetracaine hydrochloride
PC033	1-Butyl-3-sulfanilylurea, Carbutamide
PC034	Cainopan, p-Butylaminobenzoyldimethylaminoethanol
PC035	Calcium pantothenate
PC036	DL-Camphon, Campher (synth.)
PC037	Carbamazepine

PC038 Carteolol hydrochloride
PC039 Cetrimonium bromide
PC040 Chloramphenicol
PC041 Chlordiazepoxide
PC042 Chlorobutanol
PC043 4-Chloro-7-nitrobenz-2-oxa-1,3-diazole
PC044 Chloroquine diphosphate
PC045 Chlorothiazide
PC046 Chlorpromazine hydrochloride
PC047 Chlorprothixene hydrochloride
PC048 Cinchophen
PC049 Clioquinol
PC050 Clopenthixol dihydrochloride
PC051 Clorprenaline hydrochloride
PC052 Cornecaine
PC053 Cyclamic acid
PC054 Deoxycholic acid
PC055 ®-(-)-Deprenyl, Hydrochloride, Selegiline hydrochloride
PC056 Dequalinium chloride
PC057 Desalkylflurazepam
PC058 Dexbrompheniramine maleate
PC059 Dexchlorpheniramine maleate
PC060 Diazepam
PC061 Dibucaine, hydrochloride
PC062 Dichloroisoproterenol hydrochloride
PC063 Dicumarol
PC064 Diethylpropion, hydrochloride
PC065 Digoxin
PC066 1,8-Dihydroxyanthrone, Dithranol.
PC067 Diphenhydramine hydrochloride
PC068 Diphenylhydantoin, Phenytoin
PC069 Dipyrone, Metamizol sodium
PC070 Doxepin hydrochloride
PC071 Dulcin, p-Phenetylurea
PC072 Emetine dihydrochloride
PC073 Ethamivan
PC074 Ethopropazine hydrochloride
PC075 Ethyl 4-Hydroxybenzoate
PC076 Ethylnorepinephrine, 1,2-Benzenediol, 4-(2-amino-1-hydroxybutyl)-(9 Cl)
PC077 17 Alpha-ethynylestradiol
PC078 Etilefrin
PC079 Exaprolol, 2-Propanol, 1-(2-cyclohexylphenoxy)-3-[(1-methylethyl)amino]-(9 Cl)
PC080 Fenoprofen, calcium salt
PC081 Fenoterol hydrobromide
PC082 Cis(Z)-Flupentixol dihydrochloride
PC083 2-Amino-5-Chloro-2'-Fluorobenzophenone
PC084 Fluphenatzinemaleate
PC085 Folic acid
PC086 Guaifenesin
PC087 Hexobarbital

PC088 Hexoprenaline
PC089 Homatropine hydrobromide
PC090 Homotaurine
PC091 Hydrochlorothiazide
PC092 Hydrocortisone acetate
PC093 Hydroxychloroquine sulfate
PC094 p-Hydroxyephedrine
PC095 1-(4'-Hydroxy-3'-methylphenyl)-2-(tert-butylamino) ethane, hydrochloride
PC096 N-Hydroxyethylpromethazine chloride
PC097 Hydroxyzine, hydrochloride
PC098 Ibufenac
PC099 Ibuprofen
PC100 Inositol
PC101 Isoetharine
PC102 (+/-)-Isoproterenol, hemisulfate salt
PC103 Isoproterenol, hydrochloride, Isoprenaline hydrochloride
PC104 (S)-(+)-Ketoprofen, (S)-2-(3-Benzoylphenyl)-propionic acid
PC105 Labetalol hydrochloride
PC106 Lorazepam
PC107 Medazepam
PC108 Mefenamic acid
PC109 Menadione, 2-Methyl-1,4-naphtoquinone,
PC110 Mephenesin, [3-o(Tolyloxy)1,2-propanediol]
PC111 Mephentermine sulfate
PC112 Mepivacaine hydrochloride
PC113 Mesoridazine besylate
PC114 Metaraminol, Bitartrate salt
PC115 Methdilazine
PC116 Methoxamine hydrochloride
PC117 Methylatropine bromide
PC118 L-Methyldopa
PC119 Methyl p-hydroxybenzoate, Nipagin M
PC120 Metipranolol
PC121 Metronidazole
PC122 Metyprolol tartrate
PC123 Nadolol
PC124 Nadoxolol
PC125 Nalidixic acid
PC126 Naproxen
PC127 Narceine
PC128 Neomycin sulfate
PC129 Nicotinamide, Niacinamide
PC130 Nitrazepam
PC131 Nitrofurantoin
PC132 Nitrofurazone
PC133 Nordefrin, hydrochloride
PC134 Norethindrone, Norethisterone
PC135 Norfenefrine
PC136 Nortriptyline hydrochloride
PC137 Noscapine

PC138	Octopamine hydrochloride
PC139	Opipramol
PC140	Ouabain
PC141	Oxazepam
PC142	Oxprenolol, hydrochloride, 2-Propanol, 1-[(1-methylethyl)amino]-3-[2-(2-propenyloxy)phenoxy]-,hydrochloride, (2R)-(9 Cl)
PC143	Oxyphenbutazone
PC144	Cyclobarbital
PC145	Pantothenic acid, calcium salt monohydrate
PC146	Papaverine
PC147	Penbutolol
PC148	Pentobarbital
PC149	Perphenazine
PC150	Phenacetin
PC151	Phenacetin
PC152	5-Fluorouracil
PC153	Phenobarbital
PC154	Phenylbutazone
PC155	L-Phenylephrine hydrochloride
PC156	Phenyl salicylate
PC157	Pholedrine, sulfate
PC158	Phthalylsulfacetamide
PC159	Phthalylsulfathiazole
PC160	Pindolol
PC161	Quinidine
PC162	Practolol
PC163	Prednisolone
PC164	Prednisone
PC165	Primidone
PC166	Procainamide hydrochloride
PC167	Procaine hydrochloride
PC168	Prochlorperazine
PC169	Promethazine hydrochloride
PC170	Pronethalol, Alderlin
PC171	Propantheline bromide
PC172	2-(3-carboxyphenyl)propionic acid, Benzeneacetic acid, 3-carboxy-a-methyl-(9 Cl)
PC173	(+/-)-Verapamil hydrochloride
PC174	Propranolol hydrochloride
PC175	Propyphenazone
PC176	Prothipendyl hydrochloride
PC177	Protokylol hydrochloride
PC178	Pyridoxine hydrochloride
PC179	Pyrilamine maleate
PC180	Quinidine polygalacturonate
PC181	Resorcinol
PC182	Riboflavin
PC183	Saccharin sodium
PC184	Salbutamol, Albuterol
PC185	(+/-) Salbutamol, Sulfate, Albuteron 4-Hydroxy-3-hydromethylalpha[(tert-butylamino)methyl]benzyl alcohol

PC186 Salicylamide
PC187 Salicylsalicylic acid, Salicoylsalicylic acid
PC188 Santonin
PC189 Sodium phenylethylbarbiturate, Phenobarbital sodium
PC190 Sodium sulfacetamide
PC191 Sotalol hydrochloride
PC192 Sulfacetamide
PC193 Sulfadiazine
PC194 Sulfadimethoxine
PC195 Sulfaethidole
PC196 Sulfaguanidine
PC197 Sulfamerazine
PC198 Sulfameter
PC199 Sulfamethazine, Sulfamidine
PC200 Sulfamethizole
PC201 Sulfamethoxazole
PC202 Sulfamethoxypyridazine
PC203 Sulfamethylthiadiazole, Lucosil
PC204 Sulfamoxole
PC205 Sulfanilamide
PC206 N-Sulfanilyl-3.4-xylamide, Irgafen
PC207 Sulfaperine
PC208 Sulfapyridine
PC209 Sulfathiazole
PC210 Sulfisoxazole
PC211 Sulfonmethane
PC212 Talinolol, N-cyclohexyl-N'-[4-[3-[(1,1-dimethylethyl)amino]-2-hydroxyproxy]phenyl]- (9 Cl)
PC213 Temazepam
PC214 Terbutaline, Hemisulfate salt
PC215 Tetracaine hydrochloride
PC216 Theobromine
PC217 Theophylline
PC218 7-(2,3-Dihydroxypropyl)Theophylline, Dyphylline, Diprophyllin
PC219 7- (Beta-hydroxypropyl)-Theophylline, Proxyphylline
PC220 Thiamine mononitrate
PC221 Thiethylperazine
PC222 1,3-Diethyl-2-Thiobarbituric acid, Thiobarbital
PC223 Thioridazine
PC224 6-Propyl-2-thiouracil
PC225 Timolol maleate
PC226 Tinidazole
PC227 Tinopal
PC228 (+)-alpha-Tocopherol acid succinate
PC229 Tolazoline hydrochloride
PC230 Tolbutamide
PC231 Tolfenamic acid
PC232 Tranylcypromine sulfate
PC233 Triamterene
PC234 Triazolam
PC235 Trimeprazine, tartrate

PC236	Urosulfanum, Sulfaninyl urea hydrate
PC237	Xylometazoline
PC238	Chlorphentermine
PC239	Dexamethasone
PC240	Imipramine hydrochloride

Appendix 2. List of screened natural compounds.

Natural compounds (NC)

Code	Name
NC001	Acacetin
NC002	Alpha-naphthoflavone
NC003	Apigenin
NC004	L-ascorbic acid
NC005	Benzoic acid
NC006	3-Benzoylbenzo(F)coumarin
NC007	3-(2-Benzoxazolyl)umbelliferone
NC008	Bergapten, 5-Methoxypsoralen
NC009	Butylatedhydroxyanisole
NC010	Caffeic acid
NC011	(+)-Catechin
NC012	Catechol
NC013	Chrysin
NC014	o-Coumaric acid
NC015	Coumarin 102
NC016	Coumarin 30
NC017	Coumarin 7
NC018	Coumarin
NC020	Daidzein
NC021	Daphnetin
NC022	7-Diethylamino-3-thenoylcoumarin
NC023	2,3-Dihydroxybenzoic acid
NC024	2,5-Dihydroxybenzoic acid
NC025	2,6-Dihydroxybenzoic acid
NC026	3,4-Dihydroxybenzoic acid
NC027	3,5-Dihydroxybenzoic acid
NC029	2,5-Dimethyl-phenol
NC030	2,4-Dinitrophenylhydrazine
NC031	1,1-Diphenyl-2-picrylhydrazine
NC032	5-Dimethylaminonaphthalene-1-sulfonylchloride
NC033	Ellagic acid
NC034	(-)-Epicatechin
NC035	(-)-Epicatechin gallate
NC036	(-)-Epigallocatechin
NC037	(-)-Epigallocatechin gallate
NC038	Ethoxyquin
NC039	Ferulic acid
NC040	Flavon

NC041	Gallic acid
NC042	Genistein
NC043	Gitoxigenin
NC044	Gossypin
NC045	Hamamelitannin
NC046	Hesperidin
NC047	2-Hydroxyacetophenone
NC048	3-hydroxyacetophenone
NC049	4-Hydroxyacetophenone
NC050	2-Hydroxyphenylacetic acid
NC051	3-hydroxyphenylacetic acid
NC052	4-Hydroxyphenylacetic acid
NC053	Hydroquinone
NC054	Isopropyl gallate
NC055	2,4-dihydroxylbenzoic acid
NC056	Isorhamnetin
NC057	Kaempferol
NC058	Khellin
NC059	Lauryl gallate
NC060	Leucocyanidin
NC061	Luteolin
NC062	Luteolin-7-glucoside
NC063	Malvin chloride
NC064	Methyl gallate
NC065	Methyl-umbelliferone
NC066	2'-Methoxy-alpha-naphthoflavone
NC067	Morin dihydrat
NC068	Myricetin
NC070	4-Methyl pyrocatechol
NC071	Nordihydroguaiaretic acid
NC072	Octyl gallate
NC073	Phthalsäure
NC076	n-Propyl gallate
NC078	Pyrogallol
NC079	Quercetagetin
NC080	Quercetin krist.
NC082	Quercitrin dihydrat
NC083	Quercitrin dihydrat
NC084	(-)-Quinic acid
NC085	Resorcin
NC086	Resveratrol
NC087	Rhamnetin
NC088	Rosmarinic acid
NC089	Rotenone
NC090	Rutin
NC091	D(-)-Salicin
NC092	Salicylic acid
NC093	Silybin
NC094	Silybin (silymarin)
NC095	Sinapic acid

NC096	Sinigrin monohydrat
NC097	Syringic acid
NC098	Tannic acid
NC099	Camptothecin
NC100	(+)-Taxifolin
NC101	Thymol
NC102	3,4,5-Trimethoxybenzoic acid
NC103	Trolox
NC104	Umbelliferon
NC105	Vanillic acid
NC106	Vanillin
NC107	Butylatedihydroxytoluene
NC108	4-Methylumbelliferone
NC109	Isoscopoletin
NC110	Fraxetin
NC111	Fraxidin
NC112	Daphnetine
NC113	Daphnetine-7-methylether
NC114	Scopoletin
NC115	Umbelliferone
NC116	6-Methylcoumarin
NC117	4-hydroxycoumarin
NC118	3-(a-Acetonylbenzyl)-4-hydroxycoumarin
NC119	6-Methoxy-4-methylcoumarin
NC120	7-Methoxy-4-methylcoumarin
NC121	Coumarin
NC122	5,7-dihydroxy-4-methylcoumarin
NC123	6,7-dihydroxy-4-methylcoumarin
NC124	Herniarin
NC125	6,8-Dibromocoumarin carboxylic acid
NC126	Esculetin
NC127	Esculin sesquihydrate'
NC128	Citrophen
NC129	Xanthotoxin
NC130	Coumarin 106
NC131	Coumarin 153

Appendix 3. Hits from the primary screening (false positive hits (2) not included).

Compound	**Class**	**Mechanism of action**	**Primary screen** (fold of control; average ± st dev)
Acetylsulfisoxazole	Antibacterial	Sulfonamide	2.6 ± 1.2
Allobarbital	Barbiturate	$GABA_AR$ modulation	3.1 ± 1.2
Atropine methyl nitrate	Anticholinergic	Muscarinic antagonist	2.8 ± 0.8
Busulfan	Alkyl sulfonate	Antineoplastic	2.6 ± 0.6
Butethal	Barbiturate	$GABA_AR$ modulation	3.4 ± 0.9
Butidrine	Adrenergic	b antagonist	2.7 ± 0.6
Cetrimonium bromide	Topical antiseptic	Cationic surfactant	5.2 ± 1.2
Chlorothiazide	Diurete	Na^+-Cl^- Symporter inhibition	2.6 ± 0.7
Cinchophen	Analgesic	-	2.7 ± 0.8
Clopenthixol dihydrochloride	Antipsychotic	D1R/D2R antagonist	3.7 ± 0.5
Dequalinium chloride	Antiseptic	Bisquanternary quinolinium	2.6 ± 0.9
Desalkylflurazepam	Benzodiazepine	$GABA_AR$ modulation	3.5 ± 1.1
Dexbrompheniramine maleate	Antiallergic	Histamine H1R antagonist	3.4 ± 0.6
Dexchlorpheniramine maleate	Antiallergic	Histamine H1R antagonist	2.5 ± 0.7
Diazepam	Benzodiazepine	$GABA_AR$ modulation	2.9 ± 0.9
Dicumarol	Anticoagulant	Competitive inhibitor of vitamin K	0.7 ± 0.1
Folic acid	Vitamin	One-carbon metabolism	0.7 ± 0.2
Mepivacaine HCl	Local anesthetic	Na^+-channel inhibition	0.7 ± 0.1
Octopamine HCl	Biogenic amine	Adrenergic	2.1 ± 0.6
Pentobarbital	Barbiturate	$GABA_AR$ modulation	2.1 ± 0.3
Phthalylsulfathiazole	Antibacterial	Sulfonamide	2.2 ± 0.5
Selegiline HCl	Antiparkinsonian	MAO inhibition	3.5 ± 1.6
Sulfameter	Antibacterial	Sulfonamide	2.1 ± 0.2
Tolbutamide	Hypoglycemic	K^+-channel blocker	2.2 ± 0.6

Appendix 4. Compounds used in the secondary screening.

Compound	**Class**	**Mechanism of action**	**Primary screen** (fold of control; average ± st dev)	**Average effect** (n=4 repeats; fold of control)
Desalkylflurazepam	Benzodiazepine	$GABA_AR$ modulation	3.5 ± 1.1	2.1 ± 0.14
Clopenthixol dihydrochloride	Antipsychotic	D1R/D2R antagonist	3.7 ± 0.5	2.0 ± 0.21
Allobarbital	Barbiturate	$GABA_AR$ modulation	3.1 ± 1.2	1.9 ± 0.38
Butethal	Barbiturate	$GABA_AR$ modulation	3.4 ± 0.9	1.9 ± 0.12
Acetylsulfisoxazole	Antibacterial	Sulfonamide	2.6 ± 1.2	1.9 ± 0.70
Selegiline HCl	Antiparkinsonian	MAO inhibition	3.5 ± 1.6	1.8 ± 0.20
Diazepam	Benzodiazepine	$GABA_AR$ modulation	2.9 ± 0.9	1.7 ± 0.42
Sulfameter	Antibacterial	Sulfonamide	2.1 ± 0.2	1.6 ± 0.71
Dexbrompheniramine maleate	Antiallergic	Histamine H1R antagonist	3.4 ± 0.6	1.5 ± 0.71
Octopamine HCl	Biogenic amine	Adrenergic	2.1 ± 0.6	1.4 ± 0.57
Pentobarbital	Barbiturate	$GABA_AR$ modulation	2.1 ± 0.3	1.4 ± 0.28
Tolbutamide	Hypoglycemic	K-channel blocker	2.2 ± 0.6	0.9 ± 0.01
Phthalylsulfathiazole	Antibacterial	Sulfonamide	2.2 ± 0.5	0.8 ± 0.02
Folic acid	Vitamin	One-carbon metabolism	0.7 ± 0.2	0.7 ± 0.04