DYNAMIC REGULATION OF PALMITOYLTRANSFERASES BY SYNAPTIC ACTIVITY

by

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Dynamic regulation of palmitoyltransferases by synaptic activity

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Abstract

The formation and remodelling of synaptic contacts require the precise distribution and trafficking of proteins to specialized compartments. This dynamic trafficking of synaptic proteins is partly controlled by palmitoylation, which is the most common form of post-translational lipid modification in the brain. Notably, several studies have shown that synaptic proteins can be differentially palmitoylated in response to stress and synaptic activity. However, it is unclear *how* changes in synaptic activity alters protein palmitoylation. To further understand the mechanism underlying activity-induced differential palmitoylation of proteins, primary rat hippocampal cultures were used to test whether increased synaptic activity impacts transcriptional regulation or post-translational modifications of palmitoylating (zDHHCs) and depalmitoylating (ABHD17) enzymes. There were no overall changes in the transcriptional profile of the 23 DHHC enzymes nor the thioesterase, ABHD17. Post-translational modifications were not observed for zDHHC8 following increased synaptic activity. In contrast, changes were identified in the dynamic phosphorylation and/or palmitoylation of zDHHC2, zDHHC5, zDHHC6 and zDHHC9 that impact the stability or enzymatic activity of the enzymes. These modifications are likely to be important for downstream palmitoylation of synaptic proteins and the modulation of synapse plasticity.

Lay Summary

Brain development requires the formation, and refinement of connections among neuronal cells, known as “synapses”. The strengthening and weakening of synapses, called “synaptic plasticity”, is thought to be important for learning and memory. The reversible addition of lipids to the proteins, a process known as “palmitoylation”, is mediated by enzymes, called “DHHC” proteins. Palmitoylation can regulate protein function and localization. Disruption in this process can cause several brain disorders. While the role of palmitoylation in the regulation of synaptic plasticity is relatively well-studied, the mechanisms regulating the enzymes themselves are poorly understood. In this thesis, it was determined how strengthening of synapses regulates modifications to the DHHC enzymes, and the impact these modifications might have on the function of these enzymes in neuronal cells.

Preface

This thesis contains the original, unpublished work of the author, Danya Abazari. All experiments and data analysis for this manuscript were done by myself under the supervision of the senior author, Dr. Shernaz X. Bamji. The animal research contained within this thesis was performed with ethical approval from the UBC Animal Care Committee (certificates #A18-0331 and #A19-0137).

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List of Abbreviations

ABHD17	α/β hydrolase domain-containing 17 proteins
Acyl-RAC	Acyl resin assisted capture
AKAP	A-kinase-anchoring protein
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMPARs	AMPA Receptors
ANOVA	Analysis of Variance
AP5	Amino-5-phosphonovaleric acid
APT	Acyl-protein thioesterase
BSA	Bovine Serum Albumin
Ca^{2+}	calcium ion
CAMKII	Calmodulin-Dependent Protein Kinase II
CDK	Cyclin-dependent kinases
cDNA	complementary DNA
cLTP	Chemical Long-Term Potentiation
CoA	Coenzyme A
Cys	Cysteine
DHHC	aspartate-histidine-histidine-cysteine motif
DIV	Days In Vitro
DMEM	Dulbecco's Modified Eagle Medium
E	embryonic day
EDTA	Ethylenediaminetetraacetic Acid
E-LTP	Early Long-Term Potentiation
FBS	Fetal Bovine Serum
GCP16	Golgi Complex-Associated Protein Of 16kDa
GFP	Green Fluorescent Protein
Gly	glycine

GRIP1	glutamate receptor interacting protein 1
HA	Human Influenza Hemagglutinin
HEK	Human embryonic kidney
KD	Kinase-dead
L-LTP	Late Long-Term Potentiation
LTP	Long-Term Potentiation
Mg^{2+}	magnesium ion
mRNA	Messenger RNA
NMDA	N-Methyl-D-aspartic acid
OE	Overexpression
PAT	Protein Acyltransferase
PBS	Phosphate Buffered Saline
PI3K	Phosphoinositide 3-kinase
PKA	protein kinase A
PLK	Polo-like kinase
PMSF	Phenyl-Methyl Sulfonyl Fluoride
PSD-95	Postsynaptic Density Protein 95
qPCR	Quantitative Polymerase Chain Reaction
Ras	Rat sarcoma
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium-dodecyl sulfate and polyacrylamide gel electrophoresis
Ser	Serine
shRNA	short hairpin ribonucleic acid
SNARE	SNAP receptor
SPAR	Spine-associated RapGAP
TBST	Tris-Buffered Saline with Tween
Thr	Threonine
TTX	Tetrodotoxin

Tyr	Tyrosine
WT	Wild Type
zDHHC	zinc-finger DHHC (aspartate-histidine-histidine-cysteine) domain

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First and foremost, I would like to extend my deepest gratitude to my devoted supervisor, Dr. Shernaz Bamji. Thank you, Shernaz for your endless guidance and encouragement, and for being so supportive not only in research but also in life outside the lab. I am truly grateful to have the opportunity to continue my graduate career in your lab. I would also like to acknowledge my supervisory committee members, Dr. Lynn Raymond and Dr. Yu Tian Wang for all their time, valuable guidance and instruction throughout my thesis.

I am thankful to the members of Bamji Lab, past and present, for providing a friendly and exciting environment in the lab. I especially would like to thank Bhavin Shah for all the assistance and advice in the beginning when I was just getting started in the lab.

To my lovely family, thank you for all your kindness and encouragement. Thank you, mom for your never-ending love and unwavering support and for taking care of my baby Elissa, the best could happen in my life, specially during the last stretch of this work. Thank you to my brother, Foad and my beautiful sister, Elnaz, for always being there for me.

Last but undoubtedly not least, to my husband Jasem. There are no words that I can use to describe the appreciation I have for you. Thank you for the endless love, care and encouragement you have always provided me. I love you with all my heart.

Chapter 1: Introduction

1.1 The hippocampus as a model system

The hippocampus, one of a group of structures within the medial temporal lobe of the brain, is one of the most thoroughly studied areas of the mammalian central nervous system. There are two main reasons for this. Firstly, this brain region is necessary for encoding new information into long-term memory. Since the 1950s, when researchers identified the complete loss of short-term memory formation in a patient following bi-lateral removal of both hippocampi (Scoville and Milner, 1957) for the treatment of intractable epilepsy, this region of the brain has been known as a critical brain area, playing an important role in formation of new memories. A second reason for the interest in hippocampus is that it exhibits a remarkable capacity for activity-induced synaptic plasticity (Neves et al., 2008) which is believed to be required for hippocampus-dependent memory formation. In fact, 'Long-Term Potentiation' as the prototypical model of synaptic plasticity was first identified in the hippocampus (Bliss and Lømo, 1973). In addition, the simple laminar pattern of neurons and relatively well-characterized neuronal pathways, have made hippocampus an accessible and major experimental system for studying the synaptic plasticity, particularly in the context of learning and storage of memory (Neves et al., 2008). Indeed, several studies have identified learning-induced LTP-like synaptic changes in the hippocampus (Gruart et al., 2006; Whitlock et al., 2006). Finally, impaired hippocampal synaptic plasticity is thought to contribute to several brain disorders such as, Alzheimer's disease (Marchetti and Marie, 2011), Autism Spectrum Disorders (Rhee et al., 2018), and Rett syndrome (Asaka et al., 2006). This indicates the importance of understanding of the molecular mechanisms involved in synaptic plasticity that could potentially reveal underlying mechanisms of learning and memory.

Over the past half century, dissociated cultured neurons have provided an accessible and controlled environment for *in vitro* studies of the brain. Given their unique functional properties, primary cultured hippocampal neurons have also widely been used as an ideal *in vitro* model system. They present a relatively homogenous population of neurons, mainly containing approximately 90% pyramidal cells (Banker and Cowan, 1977), and a minor population (10-15%) of morphologically distinguishable inhibitory interneurons (Pelkey et al., 2017). They also exhibit other features of interest for cellular and molecular studies. For example, once dissociated, they can establish axonal and dendritic polarity (Dotti et al., 1988) and form mature functional and structural synaptic connections with one another, as well as the population of interneurons, mimicking 'natural' synapses in the hippocampus (Kaech and Banker, 2006). They can be maintained for up to months and are highly amenable to electrophysiological, genetic, and molecular manipulations as well as dynamic imaging of individual neurons and synapses. Together, primary cultured hippocampal neurons have been well established as a suitable model system for studying development, maintenance, and plasticity of excitatory hippocampal synapses. In this dissertation, primary cultured hippocampal neurons were used to elucidate the role of synaptic plasticity in the regulation of palmitoylating enzymes.

1.2 Synapse plasticity

Alteration in the strength and structure of synapses in response to neuronal activity is referred to as synaptic plasticity (Neves et al., 2008). Among all forms of activity-dependent plasticity in the brain, Long-Term Potentiation (LTP) of synaptic transmission is one of the most-studied models of synaptic plasticity. It is defined as a long-lasting strengthening of synapses based on recent patterns of activity and is thought to represent cellular correlates of

learning and memory (Martin et al., 2000). This form of plasticity was first identified in the hippocampus (Bliss and Lomo, 1973) and has been extensively characterized using different electrophysiological, biochemical, and molecular techniques (for review, see Nicoll, 2017). It is important to note that, 'LTP' is a generic term that applies to a family of plasticity-related phenomena, including tetanus-induced LTP, pairing-induced LTP, spike-timing dependent LTP, and chemically-induced LTP, each with potentially distinct expression mechanisms. However, going through each form of LTP is beyond the scope of the research presented in this work; therefore, here the focus will be on chemically-induced LTP (cLTP) which is the method used in this study to induce LTP.

cLTP is a form of NMDA receptor-dependent synaptic potentiation. *N*-methyl-D-aspartate (NMDA) receptors are glutamate-gated channels with high calcium permeability that play important physiological roles in the mammalian nervous system (Sucher et al., 1996). The activation of NMDA receptors requires the binding of two co-agonists, glycine (Gly) and glutamate (Johnson and Ascher, 1987; Kleckner and Dingledine, 1988). cLTP is induced in hippocampal neuronal cultures by brief incubation of neurons with high concentration of NMDA receptor co-agonist, Gly, in a Mg^{2+} free medium, where Gly selectively activates synaptic NMDA receptors. This effect is blocked by application of either NMDA receptor antagonist, AP5, or Ca^{2+} chelator BAPTA, indicating that influx of Ca^{2+} through NMDA receptors is required for LTP induction. The Gly-induced LTP has shown to be mediated by a rapid insertion of AMPA receptors at the surface of dendritic membrane (Lu et al., 2001).

This protocol to induce LTP created new opportunities for the study of LTP-related changes in endogenous proteins and pathways. Using monolayer dissociated neuronal cultures makes individual living cells and their synapses more accessible, and thus enables researchers to

perform both immunocytochemical and biochemical analysis on molecular and cellular changes during LTP. Additionally, while standard electrode stimulation activates only a small fraction of synapses, chemical activation of neuronal networks involves a majority of synapses, and therefore maximizes the likelihood of detection of molecular changes during and after LTP stimulation. Throughout this dissertation, this specific form of LTP will be referred to as cLTP.

1.3 Post translational modifications and regulation of synaptic plasticity

Synaptic plasticity requires precise distribution and trafficking of proteins to both pre- and post-synaptic compartments. This includes the trafficking of proteins that mediate neurotransmitter synthesis, and synaptic vesicle fusion, as well as neurotransmitter receptors, post synaptic scaffolds, and signalling proteins (Collingridge et al., 2004). For instance, during LTP, receptors from non-synaptic pools are recruited to synapses to potentiate synaptic transmission (Malinow and Malenka, 2002).

Synaptic protein trafficking, function, and localization are tightly regulated through various forms of post translational modifications (Fukata and Fukata, 2010; Globa and Bamji, 2017; Lee, 2006; Seo and Lee, 2004; Yokoi et al., 2012; Zaręba-Kozioł et al., 2018). Phosphorylation is one of the very well studied forms of these modifications (Lee, 2006; Woolfrey and Dell'Acqua, 2015). For example, phosphorylation of AMPA receptors (AMPARs) has been described as the driving mechanism of AMPAR trafficking into and out of synapses during LTP (Anggono and Huganir, 2012). Various protein kinases have been shown to be implicated in this process; these include calcium/ calmodulin-dependent protein kinase II

(CaMKII) (Hayashi et al., 2000; Lu et al., 2010), protein kinase A (PKA) (Esteban et al., 2003), and phosphatidylinositol 3-kinase (PI3K) (Man et al., 2003).

Unlike phosphorylation, little is known about lipid modifications, particularly, in the context of neuronal development and synaptic plasticity. This topic will be discussed in the subsequent sections.

1.4 Overview of protein lipidation

Lipidation is defined as the attachment of different lipid groups onto amino acid side chains of cellular proteins (Chamberlain and Shipston, 2015). There are three major forms of lipid modifications, tethering proteins toward the cytoplasmic face of cells (Fig. 1.1). These include: i) N-myristoylation defined as the attachment of 14-carbon myristic acid to an amino terminal glycine residue of the proteins through a stable amide linkage (Magee and Courtneidge, 1985), ii) prenylation, which involves the addition of 15-carbon prenyl group to a specific cystine residue in the carboxy-terminal motif through a thioester bond (Zhang and Casey, 1996), and iii) palmitoylation, which is the attachment of 16-carbon fatty acid onto cysteine residues of a protein through thioester linkage (S-palmitoylation), or amide linkage (N-palmitoylation) (Magee and Courtneidge, 1985). These lipid modifications increase protein hydrophobicity,

which in turn results in changes in different aspects of protein such as stability, trafficking, and function.

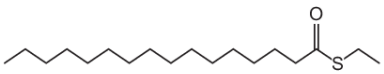
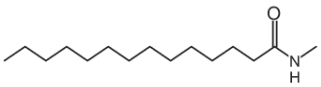
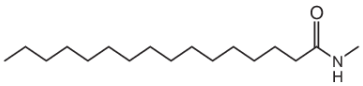
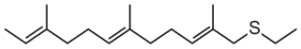
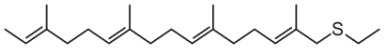
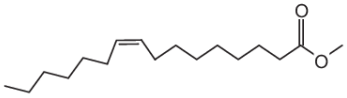
	linkage	amino acid	lipid species (major)	lipid structure	enzyme
Reversible	thioester	-Cys-	palmitate		zDHHC
Stable	amide	H_2N-Gly-	myristate		NMT
	amide	H_2N-Cys-	palmitate		Hhat
	thioether	-Cys-	farnesyl		FTase
	thioether	-Cys-	geranylgeranyl		GGTasel, GGTase II
	oxyester	-Ser-	palmitoleic		Porcn

Figure 1.1. Major lipid modifications of proteins

S-palmitoylation represents a reversible nature due to the labile thioester linkage between the lipid (most commonly, palmitate) and a Cys amino acid of a protein. Other forms of lipid modifications result in the formation of a stable bond between the lipid and the NH_2 -terminal or Cys/Ser amino acid side chains of the proteins, and thus exhibit an irreversible modification. Adopted with permission from Chamberlain and Shipston, 2015.

1.5 Protein palmitoylation

Protein S-acylation involves the addition of 16-carbon fatty-acid (typically, but not exclusively, palmitate) to selected cysteine residues via thioester bonds (S-palmitoylation), which in turn results in the increase in protein hydrophobicity and facilitates their insertion into plasma membranes (Mitchell et al., 2006). A variety of long-chain fatty acids, with different chain lengths and degrees of unsaturation have been reported to thioacylate proteins; these include arachidonate (C20:4), palmitoleate (C16:1), stearate (C18:0), and oleate (C18:1) (Liang

et al., 2002), which is in contrast to most lipid modification events, where only one or two types of lipid molecules are involved.

Unlike the other forms of lipid modifications, S-palmitoylation represents a unique reversible nature with a relatively short turnover time (Staufenbiel, 1987). In this reaction, palmitate is transferred from palmitoyl-Coenzyme A (CoA) to cysteine residues of a substrate through palmitoyl-acyltransferases (PATs), resulting in the release of a proton and a free CoA molecule. The palmitate modification can be cleaved by palmitoyl-protein thioesterases. This reversibility is what makes S-palmitoylation distinguishable from other forms of lipid modifications of cellular proteins.

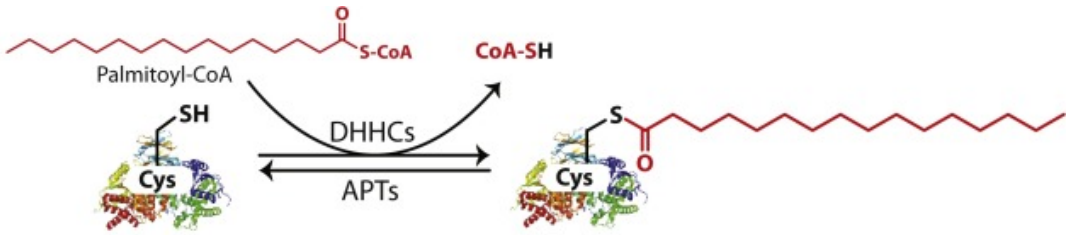


Figure 1.2. The dynamic nature of protein S-palmitoylation

In this reaction, palmitate is transferred from palmitoyl-CoA to cysteine residues of the protein through DHHC enzymes. This results in the release of a free CoA molecule. Due to the labile nature of thioester bonds between the lipid and cysteine residues, thioester bonds can be cleaved and palmitate can be removed by thioesterases. Adopted with permission from (De and Sadhukhan, 2018)

Palmitoylation-depalmitoylation cycles can be regulated by specific extracellular signals, making S-palmitoylation important not only for simple protein localization, but also for dynamic regulation of protein stability, trafficking and shuttling between intracellular compartments (Fukata and Fukata, 2010; Linder and Deschenes, 2007). Although there are other forms of palmitoylation such as N-palmitoylation (the attachment of palmitate onto selected cysteine

residues through formation of an amide bond) (Linder and Deschenes, 2007), and O-palmitoylation (the attachment of palmitate onto the hydroxyl group of serine and/or threonine residues through formation of an ester bond) (Hofmann, 2000), hereafter, the term palmitoylation refers to S-palmitoylation which is the focal point of this study.

1.6 Palmitoylating enzymes

Although palmitoylation was first identified more than 40 years ago (Schmidt and Schlesinger, 1979), the enzymes responsible for this modification have only recently been identified (Lobo et al., 2002; Roth et al., 2002). Protein palmitoylation is catalyzed by palmitoyl-acyltransferases (PATs), consisting of a family of at least 23 distinct (Fukata et al., 2004a) multi-pass transmembrane proteins, containing a conserved aspartate-histidine-histidine-cysteine (DHHC) motif (Putilina et al., 1999). The DHHC motif is located within the cysteine-rich domain of the protein, and believed to be required for the enzymatic activity of the protein (Roth et al., 2002). This motif is conserved from nematode *Caenorhabditis elegans*, and fruit fly *Drosophila melanogaster* to mammals (Putilina et al., 1999). All family members are predicted to be polytopic membrane proteins with the catalytic DHHC motif facing the cytosol (Politis et al., 2005). For clarity, the 'zDHHC' nomenclature (in which 'z' refers to a common zinc-finger domain shared among the family) will be refered to mammalian PATs in this work (see Table 1.1 for nomenclature of DHHCs).

Table 1.1. Human and mouse DHHC nomenclature

DHHC		ZDHHC			Accession Number	
Human	Mouse	Human	Mouse	Other Name	Human	Mouse
DHHC1	DHHC1	ZDHHC1	Zdhhc1		NM_013304	BC026570
DHHC2	DHHC2	ZDHHC2	Zdhhc2	REAM	BC050272	NM_178395
DHHC3	DHHC3	ZDHHC3	Zdhhc3	GODZ	NM_016598	NM_026917
DHHC4	DHHC4	ZDHHC4	Zdhhc4		NM_018106	NM_028379
DHHC5	DHHC5	ZDHHC5	Zdhhc5		NM_015457	NM_144887
DHHC6	DHHC6	ZDHHC6	Zdhhc6		BC007213	NM_025883
DHHC7	DHHC7	ZDHHC7	Zdhhc7	SERZ-β	NM_017740	NM_133967
DHHC8	DHHC8	ZDHHC8	Zdhhc8		NM_013373	AY668947
DHHC9	DHHC9	ZDHHC9	Zdhhc9		BC012826	AK032233
DHHC10	DHHC10	ZDHHC11	Zdhhc11		NM_024786	AY668948
DHHC11	DHHC11	ZDHHC23	Zdhhc23	NIDD	NM_173570	AY668949
DHHC12	DHHC12	ZDHHC12	Zdhhc12		NM_032799	BC021432
DHHC13	DHHC13	ZDHHC24	Zdhhc24		NM_207340	BC071194
DHHC14	DHHC14	ZDHHC14	Zdhhc14		NM_024630	BC059814
DHHC15	DHHC15	ZDHHC15	Zdhhc15		BC103980	NM_175358
DHHC16	DHHC16	ZDHHC16	Zdhhc16	APH2	NM_032327	XM_129300
DHHC17	DHHC17	ZDHHC17	Zdhhc17	HIP14, AKR1	NM_015336	NM_172554
DHHC18	DHHC18	ZDHHC18	Zdhhc18		NM_032283	AY668950
DHHC19	DHHC19	ZDHHC19	Zdhhc19		NM_144637	BC049761
DHHC20	DHHC20	ZDHHC20	Zdhhc20		NM_153251	AY668951
DHHC21	DHHC21	ZDHHC21	Zdhhc21		NM_178566	NM_026647
DHHC22	DHHC22	ZDHHC13	Zdhhc13	HIP14L, AKRL1	AB024495	NM_028031
–[c]	DHHC23	–[c]	Zdhhc25		–	BC049767
DHHC24	DHHC24	ZDHHC22	Zdhhc22		NM_174976	NM_001080943

The nomenclatures of DHHC are different from those of zDHHC. Adopted with permission from (Ohno et al., 2012).

Studies in yeast and some mammalian zDHHC enzymes revealed that palmitate transfer to protein substrate occurs in a two-step catalytic mechanism (Jennings and Linder, 2012; Linder and Jennings, 2013; Mitchell et al., 2010). In the first step, the cysteine residue of the

catalytically active zDHHC motif acylates, using palmitoyl-coenzyme A (CoA) as a donor, and forms a palmitoylated intermediate. In the second step, in the presence of a protein substrate, the palmitate is transferred to a cysteine residue on the substrate (Mitchell et al., 2006). While the substitution of the cysteine residue of DHH**C** with serine or alanine has shown to abolish autopalmitoylation of the enzyme, mutating the histidine residues within this motif prevented palmitate transfer, supporting this two-step mechanism (Mitchell et al., 2010; Roth et al., 2002).

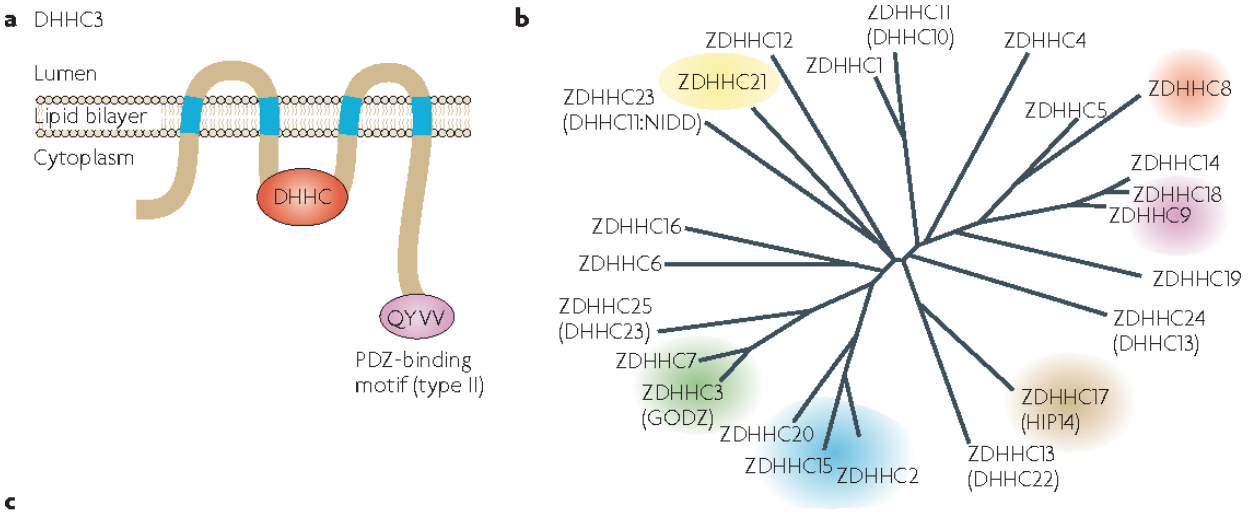
According to their phylogenetic relationship, identified zDHHC enzymes have been classified into several subfamilies (Fig. 1.3), with most containing additional interaction motifs including SRC homology 3 (SH3) domains, PSD95-DLG1-ZO1 (PDZ) binding motifs, and ankyrin repeats, which are thought to serve as key protein-protein interaction sites, directing PATs to their selective substrates (Greaves and Chamberlain, 2011; Li et al., 2010; Thomas et al., 2012). Unlike myristoylation, and prenylation, no specific consensus sequence has been identified for substrates of palmitoylation (Chamberlain and Shipston, 2015; Fukata and Fukata, 2010; Salaun et al., 2010). However, palmitoylation is predicted to frequently occur at either the terminal ends of proteins, or within cysteine-rich domains of the substrates, or adjacent to other stably linked lipid modifications such as myristoylation, and/or prenylation sites (Hu et al., 2011; Salaun et al., 2010; Zhou et al., 2006).

Different PATs appear to demonstrate unique yet partially overlapping substrate specificity. While some substrates can be palmitoylated by multiple PATs, it is also true that the palmitoylation of specific substrates relies on individual zDHHC enzymes (Fernández-Hernando et al., 2006; Fukata et al., 2004b; Greaves et al., 2010, 2010; Planey and Zacharias, 2009; Tsutsumi et al., 2009). For example, while a subset of palmitoylating enzymes including zDHHC2, zDHHC3, zDHHC7, and zDHHC15 can palmitoylate post synaptic density protein 95

(PSD-95, Fukata et al., 2004b), only zDHHC3 appears to promote AMPAR subunits palmitoylation (Sohn and Park, 2019). It is important to note that these candidate DHHC enzymes were verified by either overexpression or loss-of-function analysis. However, overexpression of enzymes or substrates may bypass DHHC regulators and could be accompanied by loss of PAT specificity. It is also true that deletion of a particular PAT may drive the palmitoylation by another PAT that would not palmitoylate the substrate under physiological circumstances. Therefore, loss-of-function studies can also be confounded by the fact that other PATs may compensate for the deleted DHHC.

The zDHHCs are localized at a variety of cellular membranes, with the majority being localized to endomembrane compartments like the ER, Golgi, or endosome membranes (Ohno et al., 2006). Several PATs (such as zDHHC2, zDHHC5, and zDHHC8) have shown to be localized at the synaptic compartments (Noritake et al., 2009; Thomas et al., 2012). Interestingly, dynamic localization of some zDHHC proteins has previously been reported. For example, following decreased synaptic activity, zDHHC2, a PAT for PSD-95, traffics to the postsynaptic membrane, where it can palmitoylate PSD-95. Palmitoylated PSD-95 in turn mediates synaptic clustering of PSD-95 and associated AMPA receptors (Noritake et al., 2009). This suggests a potential role for PATs in the homeostatic regulation of neuronal activity. The activity-induced trafficking of DHHC proteins has also been demonstrated for zDHHC5. Following increased synaptic activity, zDHHC5 can translocate to dendritic shafts, where it palmitoylates its substrate, δ-catenin. Palmitoylation of δ-catenin by zDHHC5 was shown to facilitate its trafficking into spines where δ-catenin can mediate the recruitment of AMPA receptors to the synapse (Brigidi et al., 2015).

Interestingly, among 23 zDHHC enzymes expressed in the brain, 9 have shown to be implicated in brain disorders. These include: Alzheimer's disease, Huntington's disease, schizophrenia, and intellectual disability (Cho and Park, 2016; Hornemann, 2015; Mansouri et al., 2005; Mizumaru et al., 2009; Mukai et al., 2004; Raymond et al., 2007; Sanders and Hayden, 2015; Yanai et al., 2006), all highlighting the important role of DHHC enzymes in the brain.



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DHHC	Domain structure	Substrates	Related diseases
2		PSD95, GAP43, eNOS, CKAP4, CD9, CD151 and $G\alpha_{i2}$	Colorectal cancer, hepatocellular carcinoma and non-small-cell lung cancer
15		PSD95, GAP43, CSP and sortilin	X-linked mental retardation
3		PSD95, PSD93, GAP43, $G\alpha_q$, $G\alpha_s$, SNAP25, CSP, $GABA_AR\gamma$, NCAM, eNOS, CaMK1G, GluR1 and GluR2	ND
7		PSD95, PSD93, GAP43, $G\alpha_q$, $G\alpha_s$, SNAP25, CSP, $GABA_AR\gamma$, NCAM and eNOS	ND
6		ND	ND
8		Paralemmin	Schizophrenia
9		HRAS and NRAS	X-linked mental retardation and colorectal cancer
17		SNAP25, CSP and huntingtin	Huntington's disease
21		eNOS, LCK, FYN and $G\alpha_{i2}$	ND

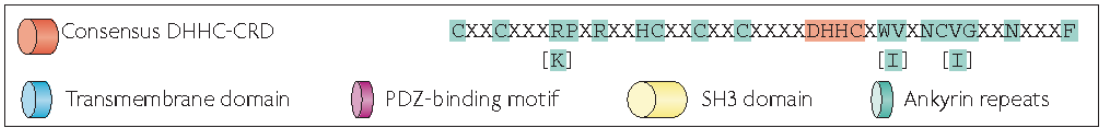


Figure 1.3. Mammalian zDHHC family, and representative enzyme-substrate pairs

(a) Schematic structure of zDHHC3, a representative PAT, consisting of four transmembrane domains with a conserved DHHC motif, facing the cytoplasmic side of the plasma membrane, and a PDZ binding motif at its C-terminus. **(b)** Phylogenetic tree of mouse zDHHCs based upon alignment of DHHC-cysteine rich domain. **(c)** Schematic representation of domain structure and identified substrates for selected DHHCs, as well as related diseases in which each DHHC is implicated. Letters highlighted in red and green denote conserved DHHC and cysteine-rich domain, respectively. Adopted with permission from Fukata and Fukata, 2010.

1.7 Depalmitoylating enzymes

As discussed earlier, due to the unstable thioester linkage, palmitoylation can reversibly control membrane association and trafficking of modified proteins. This reversibility suggests the need for specific enzymes to catalyze depalmitoylation for a complete cycle of this post translational modification. Depalmitoylation describes a process by which palmitate group is removed from palmitoylated proteins (Conibear and Davis, 2010; Linder and Deschenes, 2007). Thioesterases are the enzymes responsible for this process. Although their contribution in dynamic regulation of palmitoylation-depalmitoylation cycles are thought to be crucial (Jia et al., 2014; Koster and Yoshii, 2019), little is known about the identity of thioesterases. In fact, to date, only three classes of enzymes with depalmitoylation activity have been identified; these include: two cytoplasmic acyl-protein thioesterases (APT1, and APT2) (Hirano et al., 2009; Tomatis et al., 2010), and a lysosomal palmitoyl-protein thioesterase (PPT1) (Lehtovirta et al., 2001), as well as a novel family of α/β hydrolase domain-containing 17 proteins (ABHD17) (Lin and Conibear, 2015).

APT1, was originally isolated from rat liver, and had been characterized as lysophospholipase1 (LYPLA1) (Sugimoto et al., 1996). However, it was later shown to have higher activity as protein thioesterase (Duncan and Gilman, 1998). Although there is no clear consensus sequence for amino acid surrounding the thiol group, some degrees of specificity for APT1 have been reported. For example, caveolin, which is known to be acylated on cysteine residues of its C-terminus is not deacylated by APT1(Yeh et al., 1999), indicating a potential substrate specificity for these enzymes. In addition to APT1, vertebrates express APT2, which is a homologue of APT1 with 68% sequence identity (81% similarity) (Toyoda et al., 1999). It has been shown to depalmitoylate GAP-43 and H-Ras (Rusch et al., 2011; Tomatis et al., 2010).

Palmitoyl-protein thioesterase 1 (PPT1), was first identified based on its ability to depalmitoylate H-Ras (Camp and Hofmann, 1993). It is believed that deacylation is a process required for lysosomal degradation of palmitoylated proteins. Considering the lysosomal localization of PPT1, an important role for this enzyme in the regulation of protein turnover has been described. Traditionally, PPT1 was believed to be primarily engaged in protein degradation pathways. However, more recent data describe a subset of important roles for this enzyme in neuronal health and degeneration (Koster and Yoshii, 2019). Interestingly, some studies reported the existence of PPT1 in synaptic vesicles, demonstrating its potential role in neurotransmission (Heinonen et al., 2000; Lehtovirta et al., 2001). PPT1 was later found to regulate dendritic spines and morphology (Sapir et al., 2019). In addition, in mature neurons, SNAP25 and VAMP2 have been identified as PPT1 substrates (Kim et al., 2008), all supporting a role for this enzyme in regulation of pre and postsynaptic machinery.

ABHD17 enzymes, including ABHD17A, 17B and 17C are the most recently identified group of thioesterases, contributing to the depalmitoylation of the Ras-family of GTPases, as well as synaptic proteins. The existence of ABHD17 family of depalmitoylating enzymes was first proven by an experiment in which APT1 and APT2 were both inhibited. This inhibition was shown to have no effect on palmitoylation of N-Ras and PSD-95. However expression of ABHD17 promoted palmitate cycling on the aforementioned substrates, demonstrating the existence of a new family of thioesterases (Lin and Conibear, 2015). These enzymes have themselves shown to be palmitoylated in their N-terminal cysteine-rich domain, which is essential for plasma membrane association. It is suggested that the hydrophobicity achieved from the palmitate group places the enzymes in close proximity to the palmitoylated substrates, and therefore facilitates the enzymatic reaction.

1.8 Protein palmitoylation at synapses

S-palmitoylation is the most prominent form of S-acylation, and the most common post-translational lipid modification in the brain (Fukata and Fukata, 2010; Iwanaga et al., 2009). While post-translational phosphorylation of synaptic proteins has been well-studied and thought to play a key role in regulating synaptic plasticity (Esteban et al., 2003; Hayashi et al., 2000; Lee, 2006; Lu et al., 2010; Man et al., 2003; Woolfrey and Dell'Acqua, 2015) more recent studies have demonstrated that other post-translational modifications, including protein palmitoylation, can be equally important for the strengthening and weakening of synaptic connections (Brigidi et al., 2014; Fukata and Fukata, 2010; Globa and Bamji, 2017; Zaręba-Kozioł et al., 2018).

Palmitoylation has been shown to be implicated in diverse aspects of neuronal development and function such as neurite outgrowth, axon pathfinding, synapse development, maintenance, and plasticity (El-Husseini and Bredt, 2002; Holland and Thomas, 2017; Zaręba-Kozioł et al., 2018). Indeed, over 41% of all known synaptic proteins can be palmitoylated (Sanders et al., 2015) including ion channels (Kazim et al., 2017; Pei et al., 2018), SNARE proteins (Greaves et al., 2010; He and Linder, 2009), scaffold proteins (El-Husseini et al., 2000; Topinka and Bredt, 1998; Vallejo et al., 2017), signaling molecules (Rocks et al., 2005), and neurotransmitter receptors including AMPA, NMDA and GABA receptor subunits (Hayashi et al., 2005, 2009; Lin et al., 2009; Resh, 2006; Thomas and Huganir, 2013).

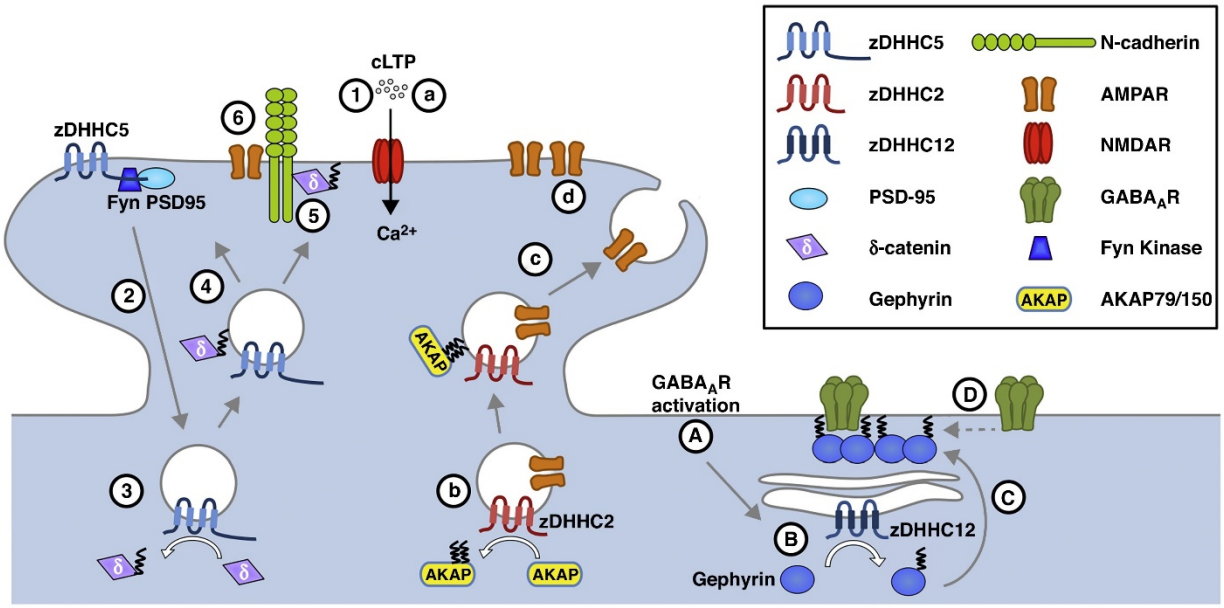
The reversible nature of palmitoylation provides an important regulatory mechanism for control of protein shuttling from and to the synapses. This includes the trafficking of a number of proteins that are critical for synapse formation and plasticity. Trafficking of ion channels (Kazim et al., 2017; Pei et al., 2018), scaffolding proteins such as PSD-95 (El-Husseini et al., 2000; Topinka and Bredt, 1998; Vallejo et al., 2017), signaling molecules such as H-, and N-Ras

(Rocks et al., 2005), neurotransmitter receptors such as AMPARs, and NMDA receptor subunits (Hayashi, 2020; Hayashi et al., 2005, 2009; Lin et al., 2009; Naumenko and Ponimaskin, 2018; Resh, 2006; Sohn and Park, 2019; Thomas and Huganir, 2013), have all been shown to be regulated by protein palmitoylation, highlighting the important role of palmitoylation in the context of neuronal biology.

1.9 Dynamic palmitoylation of synaptic substrates and synapse plasticity

The reversible nature of protein palmitoylation provides neuronal cells with an important mechanism by which they can control protein localization and function in response to neuronal stimuli, and thus coordinate synaptic plasticity. Recent studies have reported interesting results demonstrating the differential palmitoylation of a subset of synaptic proteins in response to changes in synapse activity (Fig. 1.4) (Brigidi et al., 2014; Dejanovic et al., 2014; Fukata et al., 2013; Kang et al., 2008; Noritake et al., 2009), indicating the key role of palmitoylation in the regulation of synaptic plasticity. PSD-95 is a well-studied example of this dynamic cycling. Increasing synapse activity using glutamate (which typically results in receptor internalization) has shown to be associated with PSD-95 depalmitoylation, resulting in reduction in AMPAR clustering, and a reduced amplitude and frequency of miniature EPSCs (El-Husseini et al., 2002a). Conversely, blocking synaptic activity with Tetrodoxin (TTX) has shown to induce PSD-95 palmitoylation, which in turn mediates synaptic clustering of PSD-95 and associated AMPARs (Noritake et al., 2009). In addition, palmitoylation of signaling scaffold molecule, AKAP79/150, has also been shown to be enhanced by chemical LTP stimulation, leading to its recruitment to recycling endosomes and delivery to the postsynaptic membrane. Notably, this process is disrupted in palmitoylation-deficient AKAP79/150 (Woolfrey et al., 2015). δ-catenin

is another example of a neuronal substrate which exhibits an activity-dependent dynamic palmitoylation. The palmitoylation of δ-catenin has shown to be increased upon enhanced synaptic activity, leading to its association with N-cadherin, resulting in an enlargement of postsynaptic spines which is followed by insertion of GluA1 and GluA2 subunits into the synaptic membrane (Brigidi et al., 2014).



Brigidi et al., 2014, 2015
(1) Enhanced synaptic activity
(2) Internalization of DHHC5 from dendritic spines to shafts on REs
(3) Palmitoylation of δ-catenin by DHHC5
(4) Trafficking of DHHC5 and δ-catenin to spines and insertion into membrane
(5) δ-catenin binding to cadherin
(6) Stabilization of cadherin and AMPAR at the membrane

Woolfrey et al., 2015
(a) Enhanced synaptic activity
(b) Palmitoylation of AKAP by DHHC2
(c) AKAP-mediated exocytosis of RE
(d) Increased localization of AMPAR at the postsynaptic membrane

Dejanovic et al., 2014
(A) Activation of GABA$_A$R
(B) Palmitoylation of gephyrin by DHHC12 at Golgi outposts
(C) Recruitment of palmitoylated gephyrin to the membrane
(D) Recruitment of GABA$_A$R

Figure 1.4. Palmitoylation in the regulation of activity-mediated synaptic plasticity

Schematic representation of activity-induced trafficking of zDHHC enzymes and palmitoylated substrates and their role in post synaptic receptor clustering and synapse plasticity. Adopted with permission from (Globa and Bamji, 2017)

Together, while evidence is growing that dynamic protein palmitoylation is important for synaptic plasticity, it is unclear *how* changes in synaptic activity can alter protein palmitoylation. In this study, to further understand the mechanisms regulating activity-induced differential palmitoylation of proteins, we used primary rat hippocampal cultures to examine the impact of increased synaptic activity on transcriptional regulation or post-translational modifications of zDHHC enzymes and a family of palmitoyl thioesterases, ABHD17 proteins.

There were no overall changes in the transcriptional profile of the 23 zDHHC enzymes or the thioesterase, ABHD17, nor were any changes observed in post-translational modifications of zDHHC8 following increased synaptic activity. In contrast, changes in the dynamic phosphorylation and/or palmitoylation of zDHHC2, zDHHC5, zDHHC6 and zDHHC9 were observed. Interestingly, zDHHC5 and zDHHC6 stability, and zDHHC9 enzymatic activity were seen to be impacted by synapse activity. These modifications are thought to be important for downstream palmitoylation of synaptic proteins and the modulation of synapse plasticity.

1.10 Overall Objective:

The overall objective of this study was to determine molecular mechanisms by which changes in synaptic activity translate to the differential palmitoylation of synaptic proteins to mediate synaptic plasticity.

1.11 Hypothesis:

The hypothesis of this thesis is that differential palmitoylation of synaptic substrates following altered synaptic activity is due to activity-induced changes in the transcription of zDHHC enzymes or changes in post-translational modifications of these enzymes, which in turn impacts zDHHC protein stability, trafficking or enzymatic activity.

Chapter2: Methods

2.1 DNA constructs and primers

N-terminal HA-tagged mouse DHHC 1-24, HA-tagged zDHHC5 AAA, HA-tagged P35, and Myc-tagged CDK5 plasmids were kind gifts from Dr. Gareth M. Thomas (Temple university, Philadelphia, Pennsylvania). FLAG-tagged-ABHD17A, 17B, and 17C were kind gifts from Dr. Elizabeth Conibear (University of British Columbia, Vancouver, BC). Myc-tagged PLK2, and Myc-tagged PLK2 kinase-dead mutant were kind gifts from Dr. Daniel Pak (Georgetown University, Washington, DC). shRNA against zDHHC5 was a kind gift from Dr. Richard Huganir (Johns Hopkins University, Baltimore, MD).

Table 2.1. Primers for zDHHC and ABHD17 enzymes

Primer for	Forward primer (5' to 3')	Reverse primer (5' to 3')
zDHHC1	GCT CAC CAC CTA CGA ATA CAT TGT	TGT GGG TCT CCT TTG CTT CCT
zDHHC2	GGT GAA CAA TTG TGT TGG ATT TTC	AAA GGC AGT ACA GCA GAG AGT AAG C
zDHHC3	CCA AAG GAA ATG CCA CTA AAG AG	CAC CTG CCC AGG CTT CAG
zDHHC4	CGA ACG TGT TAT TAC TGC TGC AA	GCA CCT CGA GTT CTT TGG AAA
zDHHC5	CCA AAG AAA GAG AAG ACA ATT GTA ATC A	TTA TCT GCC CAT CTG ACA CTT CTG
zDHHC6	ACA CGC GCC GGT AGG A	CCG AAT GCG AAA CCT GAT G
zDHHC7	TTG GTC TTC CTG TGC CTT GAG	CTG GGT GCC GAA CAT GAC T
zDHHC8	GTG TGT GGC TGG CCT TTT CT	ACC ACG TGG AAG CCA GTG A

zDHHC9	TCT GGA AGT CCT CAT TTG CTT CT	CAA GGA AAG TGT GAA ATC CAG TCA
zDHHC11	TGA TGC AGA CCC GAT TCC A	CTT CCG AGG AGG TAG CTC TTT CT
zDHHC12	GCG TCA ATG GGA AGA GCA A	CAG CAC CAG GAG CAG GAA AG
zDHHC13	GCT CGC AGT GCA GGA ATC A	GTG TTG ACC AAA TCC TGG AAC A
zDHHC14	CAG AAC CAA AGA AGT CAT CAT CAA TG	GGA AGA TCT TGC AGG TGA AAC AG
zDHHC15	GTT AAT AAC TGC ATT GGA TTT TCC AA	AGA CTG TTG TAG CAA TGT ACA GGC A
zDHHC16	CAT GTT GCA TGC TGT CCT CAT	TGT TGA TAT GCC TTT CGA TGC T
zDHHC17	TGC AGG CAA CCA TAG ATA CTT TAT G	AAC ACA ACC ATA AAT CAT CCA GCA
zDHHC18	CTG GCC ATC CCC ATC ATC	TCT GCA GGA GAC AGC TCA TGA C
zDHHC19	TCT TTC CCG CGG TCA CA	TGA GCG AGA CGA GAC TGA AGA A
zDHHC20	AAA TCA ACC TTT TCC TAT CAA ACC A	CAC TGA GAT TCA CTG TCC AAC AAA C
zDHHC21	TGG GTT GGC AGG CAG TTT	TCA ACA ACA AAG TGA ATC CGA AGA
zDHHC22	GGC AAG CAC TCC AGC CAT T	GTG CTG TGG GCT TAC GTT CA
zDHHC23	CGC TGA CCT TGA ACA CCA TCT	AGG ACA GTA GAA GAG GGC TGT GA
zDHHC24	CCA CCA CTG TCG CCT ATT GG	CAC AGA AAG GGC CGG TAA TTA T
ABHD17A	TCC TGT ATG GCC AGA GCA TTG	CAC TCA TAA CGT GAC GCC AGA T

ABHD17B	ACC TCG CTG CTC GGT ATG A	TCC TGA GGT CAA AGG AGA ATG AA
ABHD17C	GCA GCC GTC ATC CTC CAT	TCT GGA AAA GCA ACA CGC AAT

2.2 Antibodies

Primary antibodies used: β-actin (1:5000, Sigma A1978), anti zDHHC1 (1:1000, Abcam ab223042), anti zDHHC2 (1:1000, Santa Cruz Biotechnology sc-515204), anti zDHHC2 (1:500, Sigma SAB1101457), anti zDHHC3 (1:500, Aviva Systems Biology ARP59576), anti zDHHC3 (1:500, Sigma SAB2107413), anti zDHHC3 (1:1000, Abcam ab124084),anti zDHHC3 (1:1000, Abcam ab31837), anti zDHHC4 (1:500, Aviva Systems Biology ARP78440), anti zDHHC5 (1:1000 for WB, 5µg for IP, Sigma HPA014670), anti zDHHC6 (1:600, Abcam ab121423), anti zDHHC7 (1:500, Aviva Systems Biology OAAB11570), anti zDHHC7 (1:500, BosterBio A11785), anti zDHHC8 (1:500, Santa Cruz Biotechnology sc-374191), anti zDHHC9 (1:1000, Sigma SAB4502104), anti zDHHC9 (1:1000, Thermo Fisher Scientific PA5-26721), anti zDHHC11 (1:500, Abcam ab116065), anti zDHHC12 (1:500, Aviva Systems Biology ARP60674), anti zDHHC13 (1:500, Aviva Systems Biology ARP44398), anti zDHHC14 (1:500, Aviva Systems Biology ARP42628), anti zDHHC15 (1:500, Sigma SAB4500608), anti zDHHC15 (1:200, Abcam ab121203), anti zDHHC15 (1:500, Santa Cruz Biotechnology sc-169847), anti zDHHC15 (1:500, Thermo Fisher Scientific PA5-39327), anti zDHHC16 (1:500, Aviva Systems Biology ARP50063), anti zDHHC17 (1:300, Proteintech 15465-1-AP), anti zDHHC17 (1:500, Sigma AV47141), anti zDHHC18 (1:1000, Abcam ab154790), anti zDHHC19 (1:500, Abcam ab179545), anti zDHHC20 (1:500, Aviva Systems Biology ARP72069), anti zDHHC21 (1:300, Abcam ab103755), anti zDHHC22 (1:500, Santa Cruz

Biotechnology sc-514005), Phospho-PLK Binding Motif (ST*P) (1:1000, Cell Signaling Technology 5243S), anti-HA (1:1000, Cell Signaling Technology C29F4), anti-myc (1:1000, Cell Signaling Technology 2276), anti-GFP (1:3000, Abcam ab290), anti ABHD17 (1:1000, Origene TA331704), anti ABHD17 (1:1000, Proteintech 15854-1-AP), anti FLAG (1:1000, Sigma F7425), PSD-95 (1:500, Abcam, ab2723).

Secondary antibodies used: Goat anti-mouse IgG-HRP (1:6000, BioRad 170-6516), Goat anti-rabbit IgG-HRP (1:6000, BioRad 170-6515)

2.3 Cell culture

Primary hippocampal neurons: Hippocampi from embryonic day 18 (E18) Sprague Dawley rats of either sex were prepared as previously described (Xie et al., 2000). Briefly, hippocampi were dissected, and incubated with 0.25% Trypsin (Thermo Fisher Scientific) and 0.05% DNase (Thermo Fisher Scientific) for 20 and 3 minutes, respectively. Cells were dissociated with titration and plated at a density of 3.2 million/10-cm culture dish for biochemical assays. Cells were allowed to adhere in plating media containing Minimum Essential Media (MEM; Gibco, Thermo Fisher Scientific), supplemented with 10% (vol/vol) heat-inactivated-fetal bovine serum (FBS) (Gibco, Thermo Fisher Scientific), sodium pyruvate (Gibco, Thermo Fisher Scientific), 0.5% glucose, GlutaMAX (Gibco, Thermo Fisher Scientific), and Pen/Strep (Gibco, Thermo Fisher Scientific). After 3 hours plating media was replaced with maintenance media containing Neurobasal medium (Gibco, Thermo Fisher Scientific), supplemented with NeuroCult SM1 (StemCell, instead of B27 in the original protocol), GlutaMAX (Gibco, Thermo Fisher Scientific), and Pen/Strep (Gibco, Thermo Fisher Scientific). Cultures were maintained at 37°C and 5% CO2.

HEK Cells: HEK293T cells (Sigma) were aliquoted into a 10 cm culture dish with 15 ml of pre-warmed (37°C) DMEM (Gibco, Thermo Fisher Scientific), supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (FBS) (Gibco, Thermo Fisher Scientific), 1% Pen/Strep. HEK Cells were maintained in an incubator at 37°C and 5% CO2.

2.4 Transfection

Primary hippocampal cultures - transient transfections: Neurons were transfected at 9-11 days in vitro (DIV) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol, and used for experiments on DIV 12-15.

Primary hippocampal cultures – Amaxa nucleofection: Neurons were nucleofected with identified plasmids prior to plating at 0 DIV using Amaxa Rat Neuron Nucleofector kit (DGP-1003; Lonza) according to manufacturer's protocol. Cells were then used for experiment at 13-15 DIV.

HEK Cells: HEK293T Cells were transfected at 70-80% confluency, using Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendations and used for experiments 24-48 hours after transfection.

2.5 Neuronal stimulation (cLTP)

Neuronal activity was enhanced as per previously published protocol (Lu et al., 2001). In short, at 13-15 days in vitro, the maintenance media was removed and stored at 37°C and cells were washed 3 times with pre-warmed (37°C) Mg^{2+} -free extracellular solution made of NaCl 140 mM, $CaCl_2$ 1.3 mM, KCl 5.0 mM, HEPES 25 mM, glucose 33 mM, supplemented with TTX 0.0005 mM and strychnine 0.001 mM (pH 7.4). To chemically induce LTP, cells were incubated with the same media supplemented with 200 μM glycine for 3 minutes. Cells were then washed

with regular extracellular solution containing 2 mM $MgCl_2$.The solution was then replaced with stored maintenance media. Neuronal cells were maintained in 37°C incubator with 5% CO_2 for the indicated time prior to experimentation. Control cells were subjected to the same number of washes using the same solutions as the experimental groups but were not exposed to glycine during the 3-minute incubation.

2.6 Immunoblot assay

Brain tissue, primary hippocampal neurons, and HEK293T cells were washed with ice-cold PBS and lysed in ice-cold Tris Lysis Buffer containing 1% IGEPAL (Sigma), 50mM Tris-HCl pH 7.5, 150mM NaCl, 10% Glycerol, supplemented with phenylmethanesulfonyl fluoride solution (PMSF) and a protease inhibitor cocktail with EDTA (Roche). The samples were vortexed, and run through a 26-gauge syringe and kept at 4°C to nutate for 30 minutes. Lysates were then cleared by spinning down at 16,000 x g for 30 minutes at 4°C. Protein quantification was performed using a BCA assay kit (Thermo Scientific) as per the manufacturer's instructions. Proteins were separated by electrophoresis on a 10-12% SDS-PAGE gel. Proteins were then transferred to a PVDF membrane (BioRad) and blocked for one hour in 3-5% BSA in TBST. The membrane was then incubated overnight at 4°C with identified primary antibody. The membranes were then washed 3 times for 15 minutes in TBST at room temperature with agitation and incubated with appropriate secondary antibodies for 1 hour at room temperature, before being washed 3 times for 15 minutes with TBST. Proteins were visualized using chemiluminescence (Immobilon Western Chemiluminescent HRP Substrate, Millipore, #WBKLS0500) on Bio-Rad ChemiDoc (XRS+). Blots were quantified using Image J software. For reprobing, blots were stripped as per previously published protocol (Yeung and Stanley, 2009).

2.7 Immunoprecipitation

The Cells were lysed as described above and incubated overnight at 4°C with identified antibodies, under gentle rotation. 80-100 µL of a mix of protein A and G- Agarose (Roche) was added to the samples, and the beads were recovered after 4 h, before being washed 5 times with cold Tris Lysis Buffer. Proteins were eluted from the beads by heating in 2X SDS loading buffer for 5 min at 80°C. samples were analyzed by SDS-PAGE, then immunoblotted with identified antibodies.

2.8 RNA isolation and qPCR

At 15 DIV hippocampal cultured neurons were stimulated as described above and mRNA was isolated after identified time points using TRIzol Reagent (ThermoFisher Scientific) as described by manufacturer's instructions. 200 ng of total DNA-free RNA was reverse transcribed using Verso cDNA Synthesis Kit (Thermo Scientific). The cDNA was then quantified by qPCR using SYBR green (ThermoFisher Scientific). Real-time quantitative PCR (qPCR) analysis was performed at the Biomedical Research Center at UBC using a 7900HT Real-Time PCR thermocycler machine (Applied Biosystems).

mRNA levels of genes of interest were normalized to GAPDH and shown as fold change over baseline using the delta-delta CT method (Schmittgen and Livak, 2008).

2.9 Acyl-Rac Assay

Protein palmitoylation assay was performed using CAPTUREome S-palmitoylated protein kit (Badrilla, Leeds, UK), as described by manufacturer's protocol, with the following modification: Protein concentration was measured after dissolving the precipitated protein, to ensure starting with equal protein concentrations. In short, cells were lysed and incubated with blocking reagent

to block all free thiol groups. Extracted proteins were then acetone precipitated. Pellets were re-dissolved and protein concentration was measured using BCA assay. The palmitate groups on proteins were cleaved using thioester cleavage reagent. Proteins with newly liberated thiols were then captured using CAPTUREome resin. Captured proteins were then eluted from resin. samples were analyzed by SDS-PAGE, then immunoblotted with identified antibodies.

2.10 PhosphoProtein Purification Assay

Protein phosphorylation assay was performed using PhosphoProtein Purification Kit (Qiagen), exactly as described by manufacture's guideline. For negative control, the lysates were incubated with 800 units of lambda protein phosphatase (New England Biologicals) for 45 minutes at room temperature.

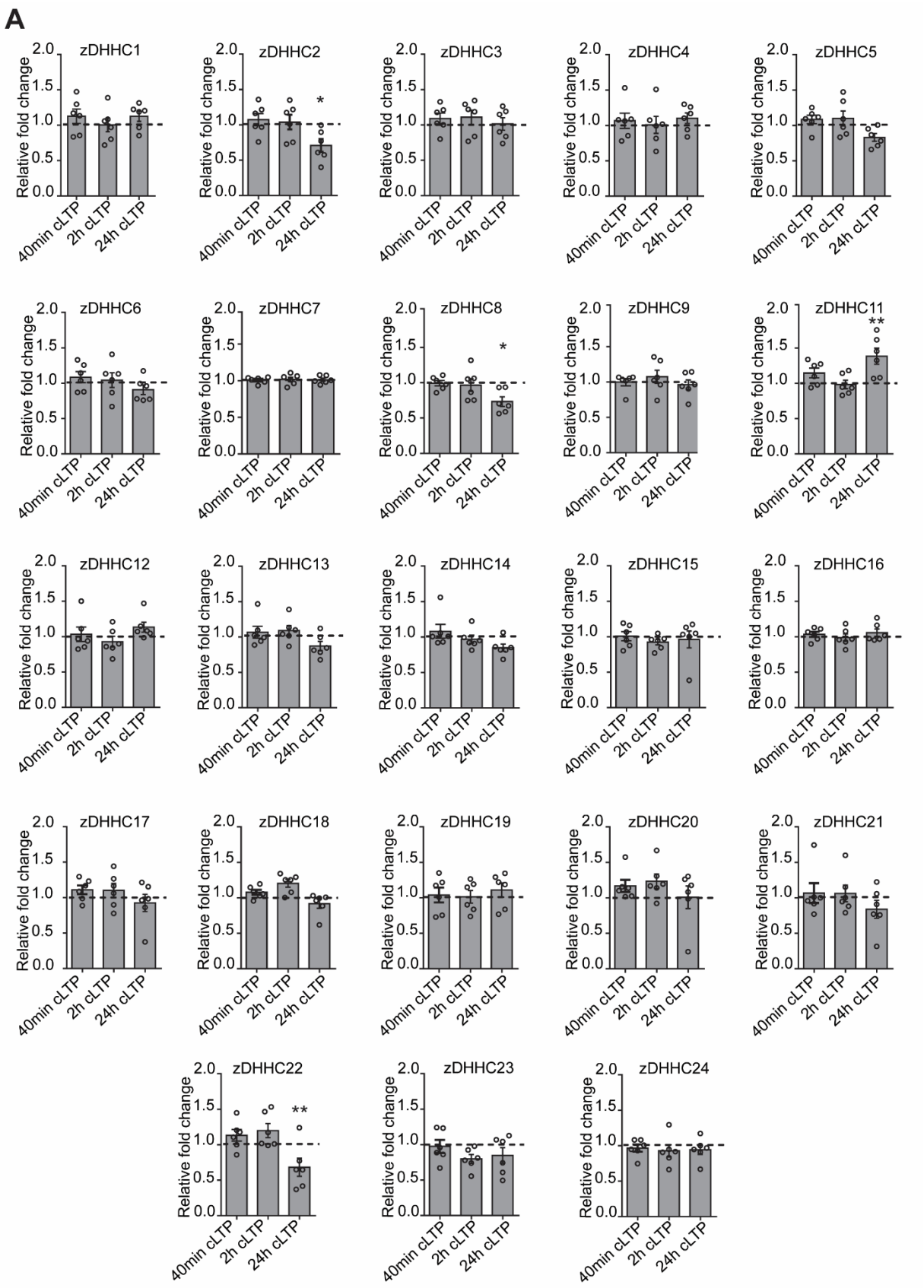
2.11 Statistical Analysis

All data values are expressed as means ± SEM. For all experiments, the value of 'n' refers to the number of separate cultures. Statistical significance was measured using either unpaired T-test or One-way ANOVA (with Dunnett's multiple comparisons, or Tukey's multiple comparisons) where applicable and defined when $p < 0.05$. In all figures, * = $p < 0.05$, ** = $p < 0.01$, and *** = $p < 0.001$. All statistical analysis was performed in GraphPad Prism (La Jolla, CA, USA). Figures were generated using Adobe Illustrator CS6 software (Adobe Systems Inc., San Jose, CA).

Chapter 3: Results

3.1 zDHHC and ABHD17 mRNA levels are largely unchanged after cLTP

Studies on various forms of synaptic plasticity provide strong evidence describing the impact of synaptic activity on transcriptional profiles of proteins (Costa-Mattioli et al., 2005; Engelmann and Haenold, 2016; McClung and Nestler, 2008; Tabuchi, 2008). Long-term potentiation, in particular, has shown to alter mRNA expression of several proteins (Roberts et al., 1998; Tzingounis and Nicoll, 2006). Therefore, in order to investigate the impact of synaptic activity on expression of all 23 zDHHC palmitoylating enzymes, as well as depalmitoylating enzymes, ABHD17A, 17B, and 17C, mRNA transcripts were quantified in cultured hippocampal neurons. In order to increase network activity, a well-established (Brigidi et al., 2015; Lu et al., 2001; Woolfrey et al., 2015) glycine-based cLTP protocol was used. 14-15 days in vitro (DIV) cultured hippocampal neurons were briefly (3 min) incubated with high concentration (200 μM) of glycine in the presence of glycine receptor blocker, strychnine, to avoid the potential activation of glycine receptors. This has been shown to recruit AMPARs to the synaptic membrane and enhance synapse strength (Lu et al., 2001). mRNA was then isolated 40 minutes, 2 hours, and 24 hours after glycine treatment, and results were compared with control (untreated), and mock-treated conditions, and measured with qRT-PCR. While cLTP resulted in no changes in the mRNA levels of the majority of zDHHCs and ABHD17 enzymes (Fig. 3.1 A, B), it reduced the number of mRNA transcripts for zDHHC2, zDHHC8, and zDHHC22, 24 hours following glycine treatment. zDHHC11 mRNA levels, however showed an increase at this time point, suggesting a modest bidirectional regulation of zDHHC mRNAs by cLTP.


A
zDHHC1
zDHHC2
zDHHC3
zDHHC4
zDHHC5
zDHHC6
zDHHC7
zDHHC8
zDHHC9
zDHHC11
zDHHC12
zDHHC13
zDHHC14
zDHHC15
zDHHC16
zDHHC17
zDHHC18
zDHHC19
zDHHC20
zDHHC21
zDHHC22
zDHHC23
zDHHC24
Relative fold change
2.0
1.5
1.0
0.5
0.0
40min cLTP
2h cLTP
24h cLTP
*
**

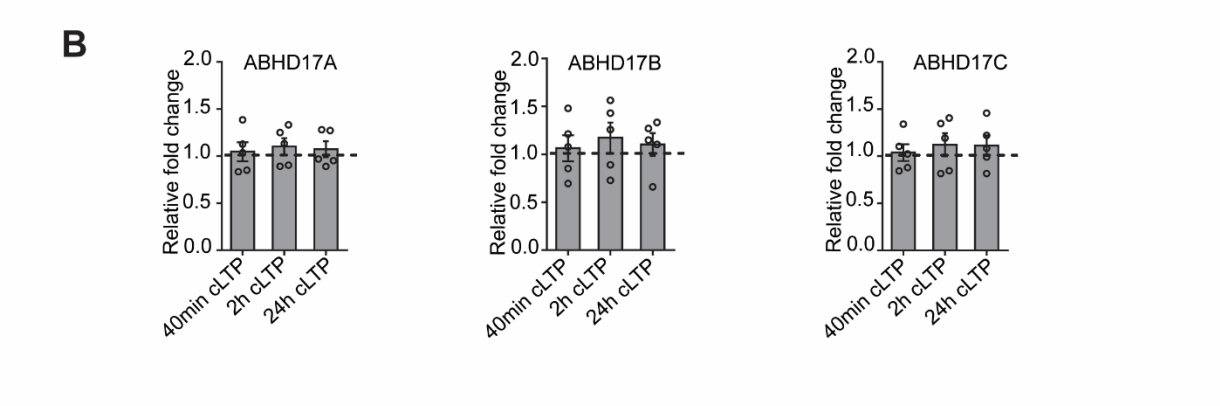
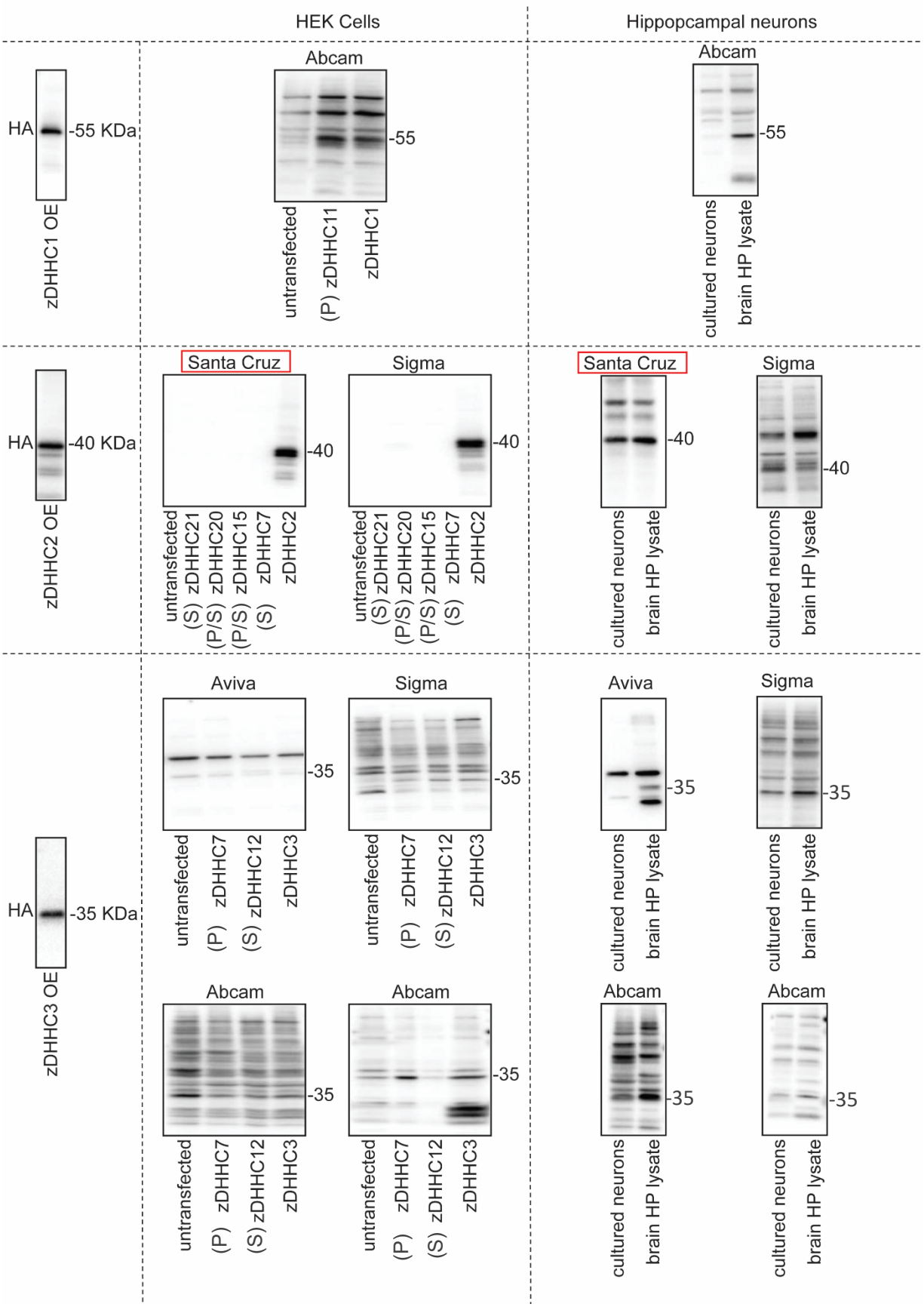


Figure 3.1. cLTP does not alter mRNA levels of the majority of zDHHC and ABHD17 enzymes

(A) Real-time qPCR quantitation of 23 mammalian zDHHC mRNA levels and **(B)** ABHD17 mRNA levels following cLTP treatment. The relative levels of mRNA for each zDHHC and ABHD17 extracted from 14-15 DIV cultured rat hippocampal neurons following cLTP induction were normalized to the mRNA levels of mock treatment and then internal control, GAPDH. Values are means ±SEM (N=6, N refers to the number of separate cultures) expressed as fold change of mRNA expression at different time points after cLTP treatment relative to untreated control. *P<0.05, **P<0.01 (One-way ANOVA followed by Dunnett's multiple comparisons).

Next, any changes in post-translational modifications of zDHHC proteins following activity were investigated, along with the impact that these modifications might have on the stability of zDHHC enzymes. To do this, antibodies for all zDHHCs were first validated. Almost all commercially available antibodies for these enzymes were obtained, and the specificity of each antibody was tested. Each zDHHC enzyme tagged with an HA epitope. was transfected into HEK 293T cells, while the closest phylogenetic or structural zDHHC for each enzyme was also transfected into HEK 293T cells in parallel. The cells were then lysed, and the efficacy and specificity of each antibody tested using western blotting. Untransfected HEK cells were used as a negative control. Antibodies were also tested against endogenous proteins in lysates from either rat cultured hippocampal neurons or rat hippocampus (Fig. 3.2).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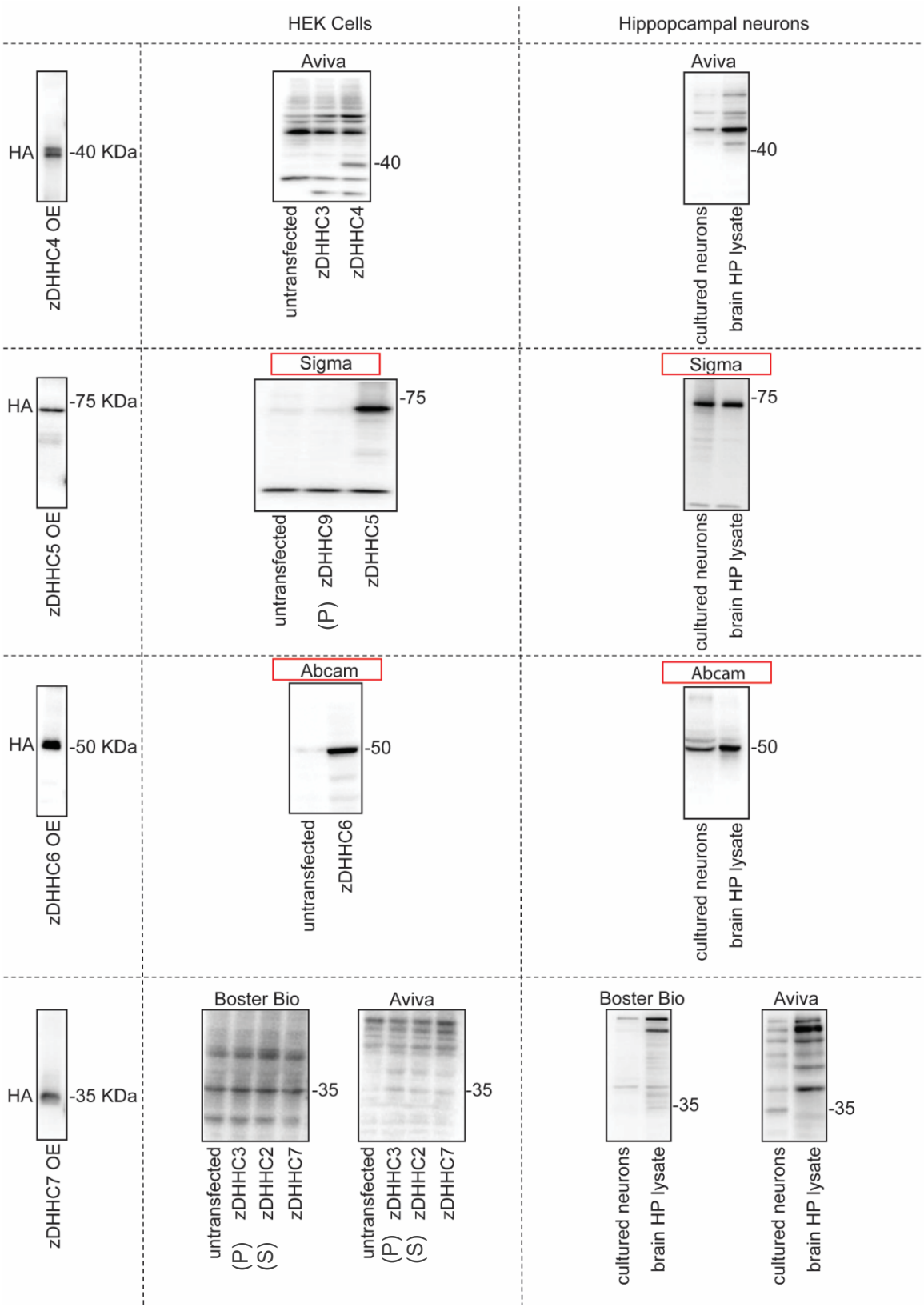
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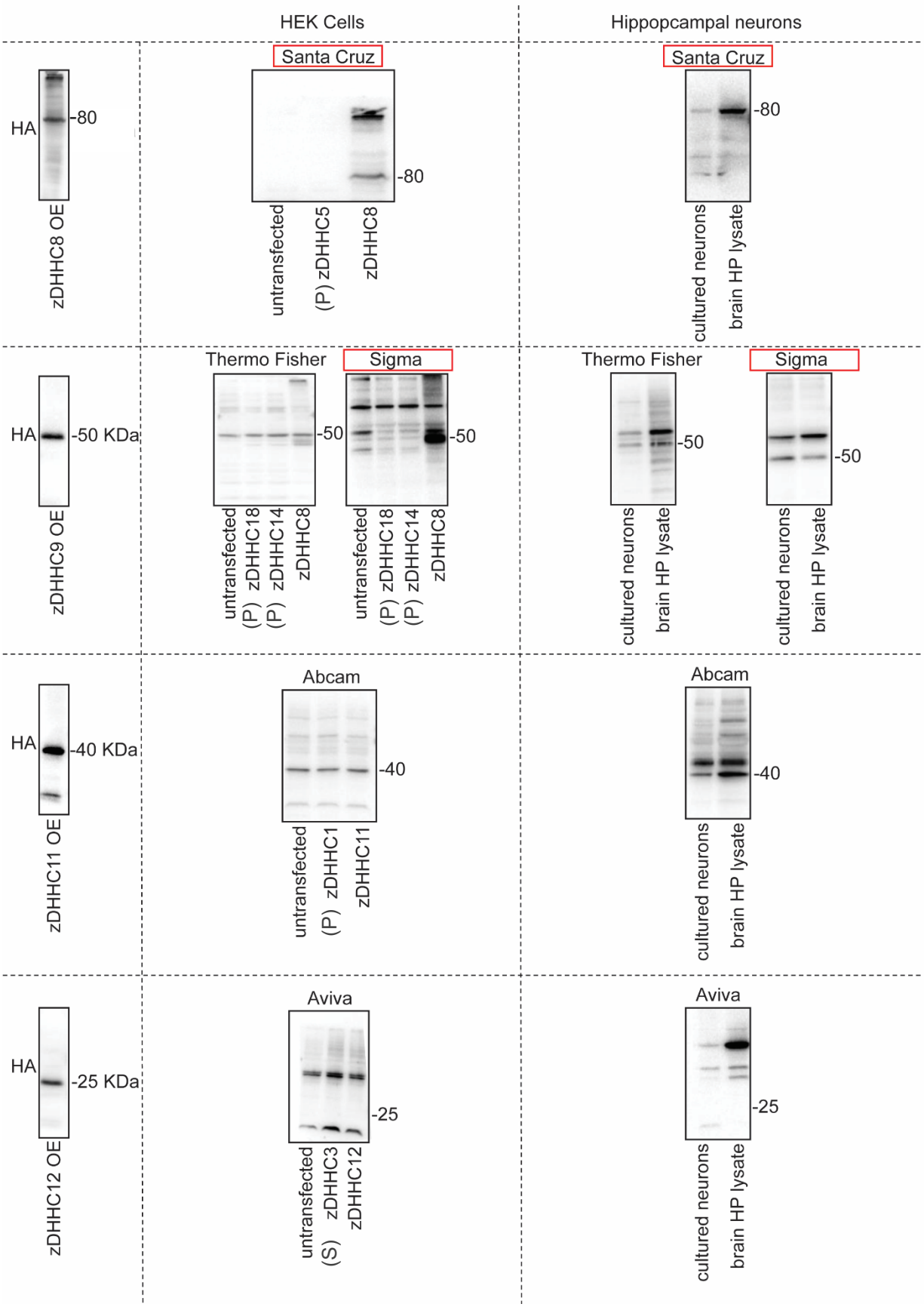
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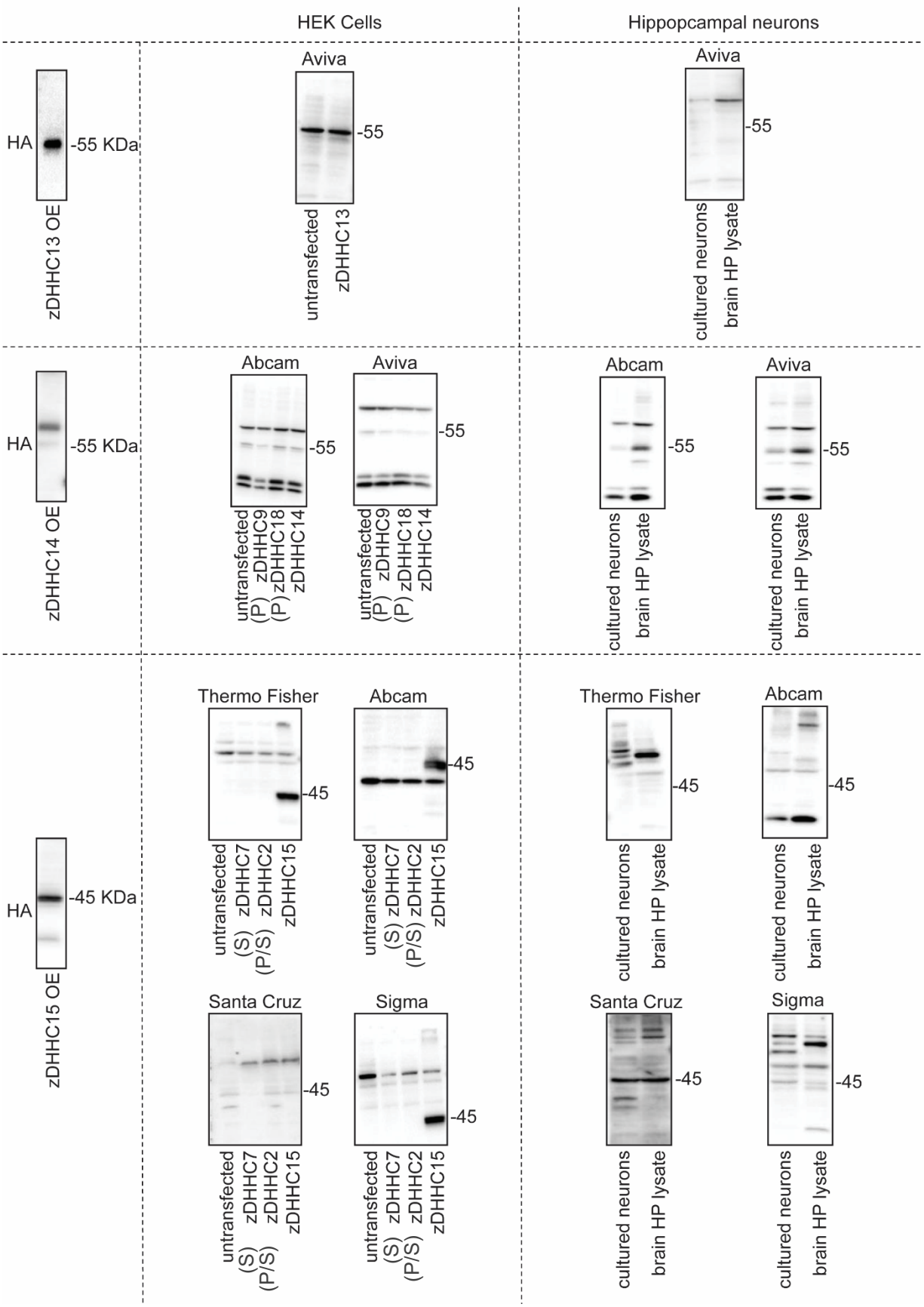
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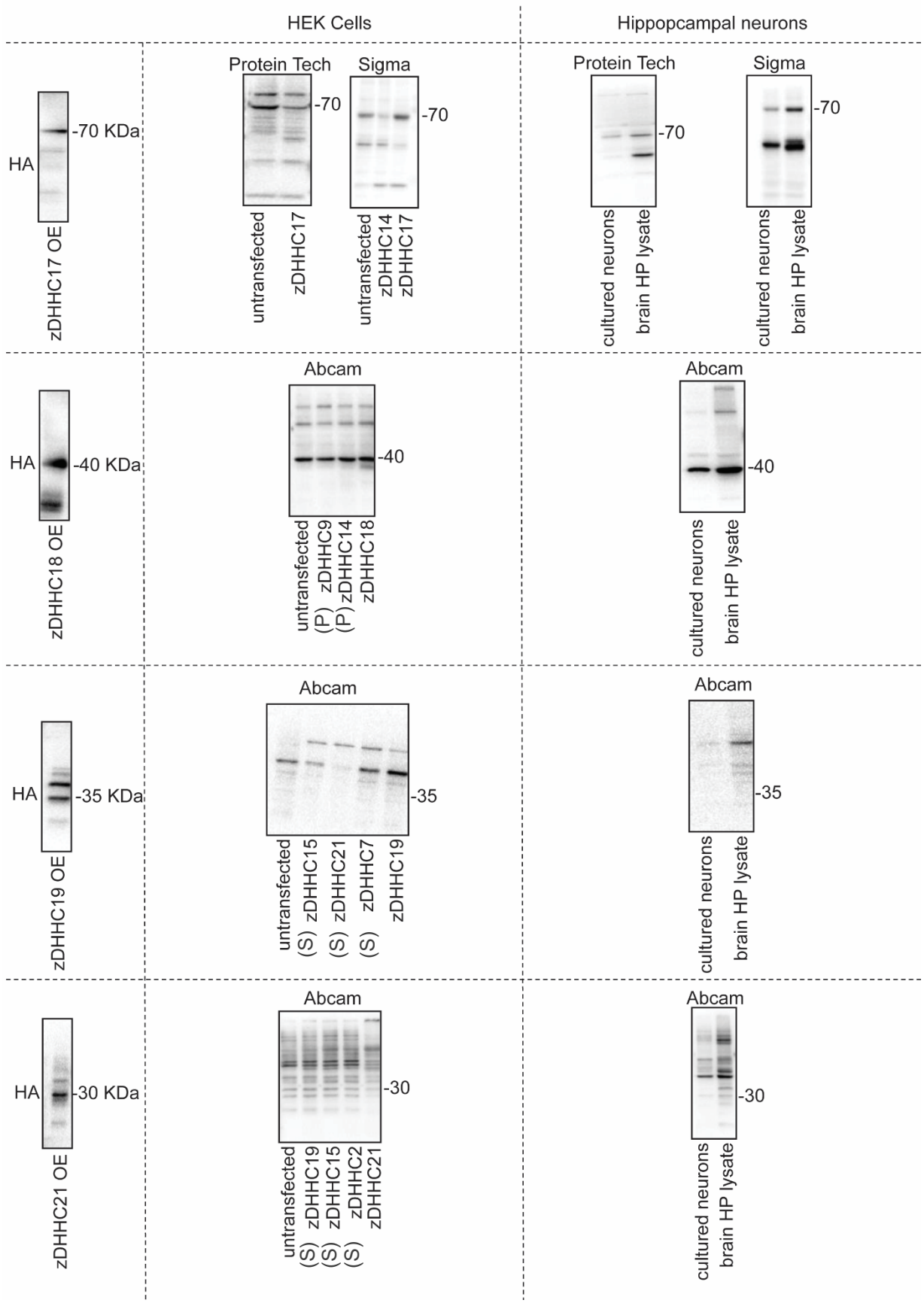
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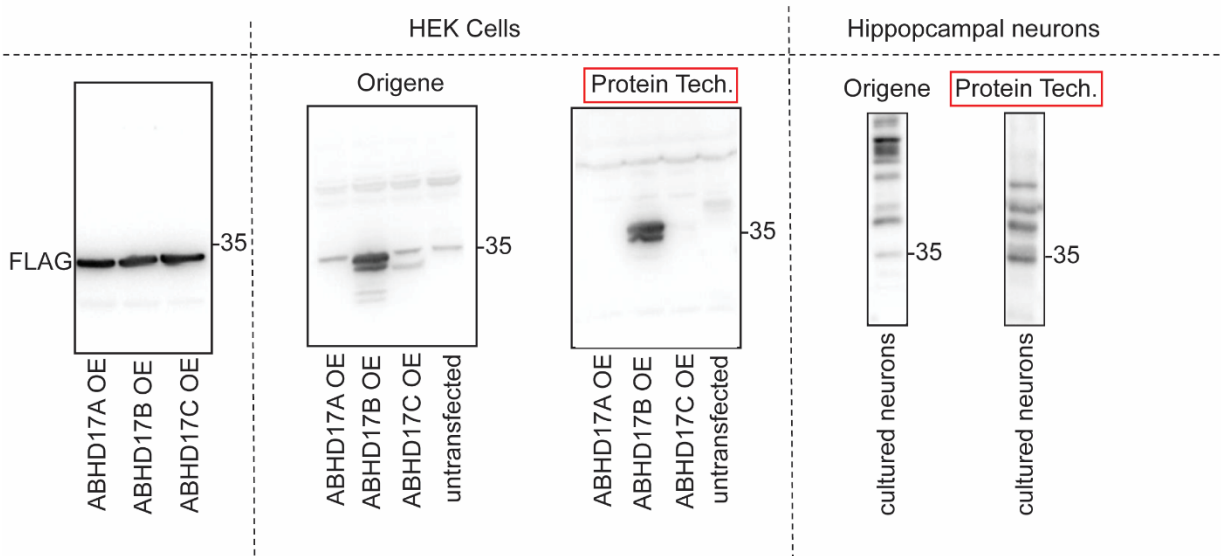


Figure 3.2. Validation of commercially available zDHHC antibodies

Representative images of western blots testing efficacy and specificity of zDHHC and ABHD17 antibodies. *Left:* HEK cells expressing the indicated tagged zDHHC or ABHD and probed for the tag to demonstrate protein expression. *Middle:* HEK cells expressing the indicated tagged zDHHC and probed with an antibody from the indicated company. To ensure antibody specificity, the zDHHC closest to the one being tested both phylogenetically (indicated by P) and structurally (indicated by S) was included. *Right:* Ability of the antibodies to detect endogenous zDHHCs or ABHDs in rat primary hippocampal cultures or hippocampal lysates. Transfection of zDHHC16, 20, and 23 was unsuccessful. Among all tested antibodies only five (marked with red boxes) were shown to be specific for the target proteins.

After testing the specificity of almost all commercially available zDHHC antibodies, only five of them were shown to be specific for the indicated zDHHC (zDHHCs 2, 5, 6, 8 and 9). These five enzymes were selected for subsequent analysis in this study. Notably, zDHHC2 and 5 have previously been shown to regulate activity-induced palmitoylation of synaptic proteins (Brigidi et al., 2014; Noritake et al., 2009), and zDHHC8 and zDHHC9 have been shown to be mutated in patients with schizophrenia (Faul et al., 2005; Mukai et al., 2004) and X-linked intellectual disability (Mitchell et al., 2014; Shimell et al., 2019), respectively. ABHD17 antibodies were also validated (Fig. 3.2), demonstrating that the antibody tested recognizes the ABHD17B isoform. The effects of synaptic activity on protein levels, phosphorylation and

palmitoylation of each of these five DHHC enzymes was investigated to explore the potential mechanisms by which synaptic activity can regulate the dynamic palmitoylation of zDHHC protein substrates.

3.2 cLTP downregulates phosphorylation of zDHHC2

First, it was determined if synaptic activity can alter zDHHC2 post translational modifications. Lysates of cultured hippocampal neurons were assayed for changes in palmitoylation of zDHHC2 using an acyl-RAC palmitoylation assay. No changes were observed in zDHHC2 palmitoylation either 10 minutes, 40 minutes, and 24 hours post-cLTP treatment (Fig. 3.3 C, D). On the other hand, zDHHC2 phosphorylation was significantly reduced 40 minutes, and 24 hours after cLTP induction (Fig. 3.3 E, F). Notably, activity-induced trafficking of zDHHC2 has shown to be important for dynamic palmitoylation of synaptic substrate PSD-95 (Noritake et al., 2009). It would be worthwhile to see whether the observed decreased phosphorylation is required for changes in zDHHC2 trafficking and subsequent changes in PSD-95 palmitoylation. This possibility will be further discussed in chapter 4.

Protein phosphorylation has previously been shown to impact protein stability (Ang et al., 2008; Nishi et al., 2014). Therefore, we investigated whether changes in phosphorylation affect stability of zDHHC2 protein. Unlike what was observed with zDHHC2 mRNA profile 24 hours after cLTP induction (Fig. 3.1 A) no changes were observed in zDHHC2 protein levels 40 minutes, 2 hours and 24 hours after cLTP treatment (Fig. 3.3 A, B).

Considering the reversible nature of palmitoylation, dynamic palmitoylation of PSD-95 can be also explained through activity-induced changes in thioesterases. Recent work has

identified ABHD17 as the only thioesterase to palmitoylate PSD-95 (Yokoi et al., 2016). Therefore, the effects of increased synaptic activity on ABHD17 post-translational modifications, stability and gene expression were investigated. No changes were observed in palmitoylation or phosphorylation of ABHD17 following activity (Fig. 3.3 I-L). Similarly, cLTP did not affect mRNA levels (Fig. 3.1 B) and protein stability of ABHD17 (Fig. 3.3 G, H).

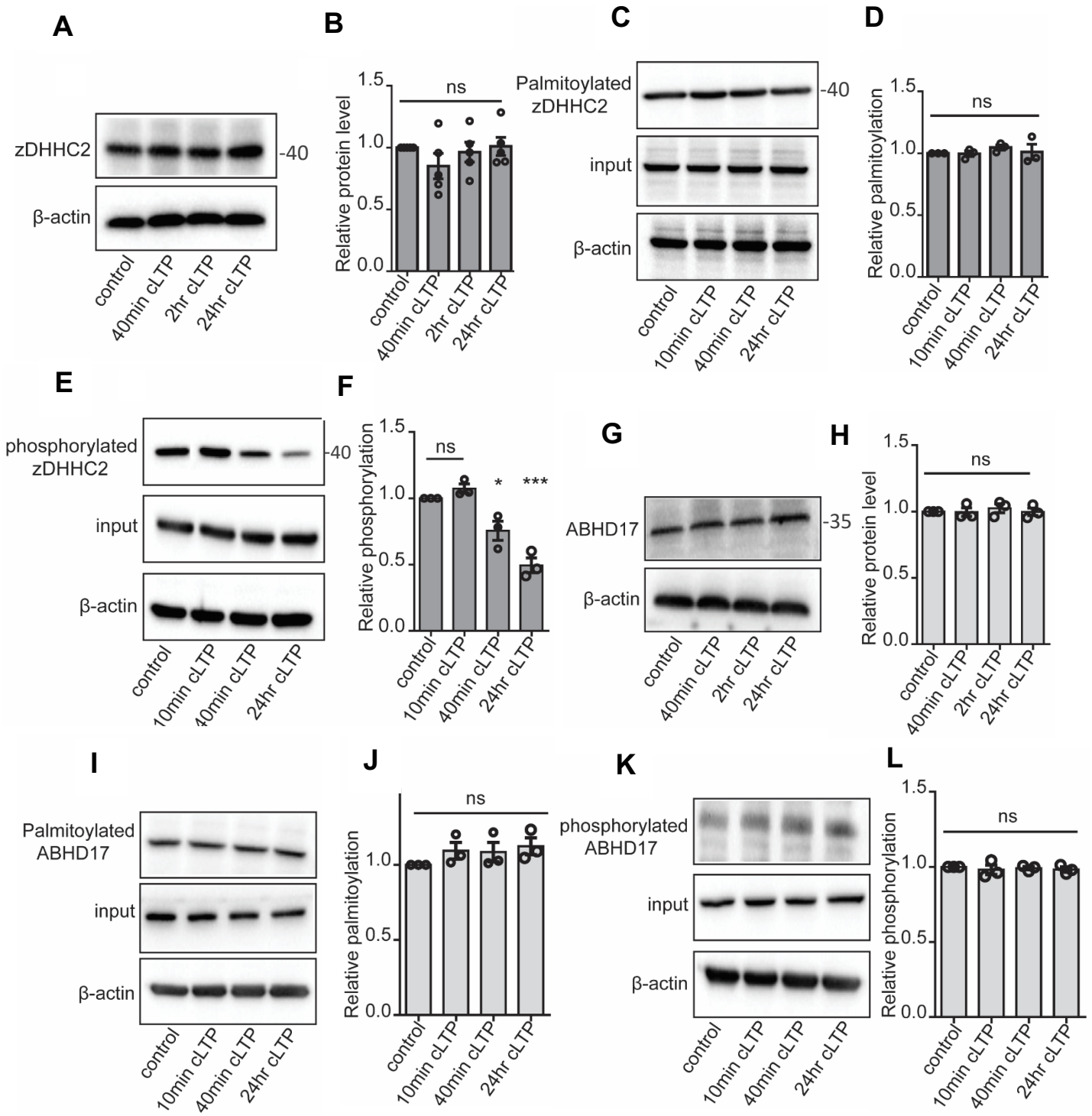


Figure 3.3. cLTP decreases the phosphorylation of zDHHC2

(A) Western blot analysis of changes in zDHHC2 protein levels in 14-15 DIV cultured hippocampal neurons after chemically induction of LTP at different time points. **(B)** Bar graph represents changes in zDHHC2 protein levels following increase in synapse activity relative to untreated control. Increased synaptic activity does not alter total levels of zDHHC2 protein (N=5 separate blots from 5 separate

Figure 3.3. continued:

cultures). **(C)** Acyl-Rac assay from 14-15 DIV cultured hippocampal neurons. Palmitoylated proteins were purified using Acyl-Rac assay, and were subjected to SDS-PAGE followed by blotting with zDHHC2 antibody. There were no changes in palmitoylated zDHHC2 following cLTP treatments. **(D)** Graph represents changes in palmitoylation of zDHHC2 protein following cLTP relative to control condition (N=3, separate blots from 3 separate cultures). **(E)** Phospho-protein purification assay from 14-15 DIV cultured hippocampal neurons. Phosphorylated proteins were separated by SDS-PAGE, and blots were probed with zDHHC2 antibody. As shown in panel **(F)** Overall phosphorylation levels of zDHHC2 declined 40 minutes and 24 hours after cLTP induction (N=3, separate blots from 3 separate cultures). **(G-L)** Synapse activity did not impact protein levels, palmitoylation and phosphorylation of ABHD17 enzymes (N=3, separate blots from 3 separate cultures). Values represent means ±SEM, *P<0.05, **P<0.01, ***P<0.001, One-way ANOVA followed by Dunnett's multiple comparisons.

3.3 cLTP decreases zDHHC5 stability via phosphorylation of Ser/Thr in Polo box motif

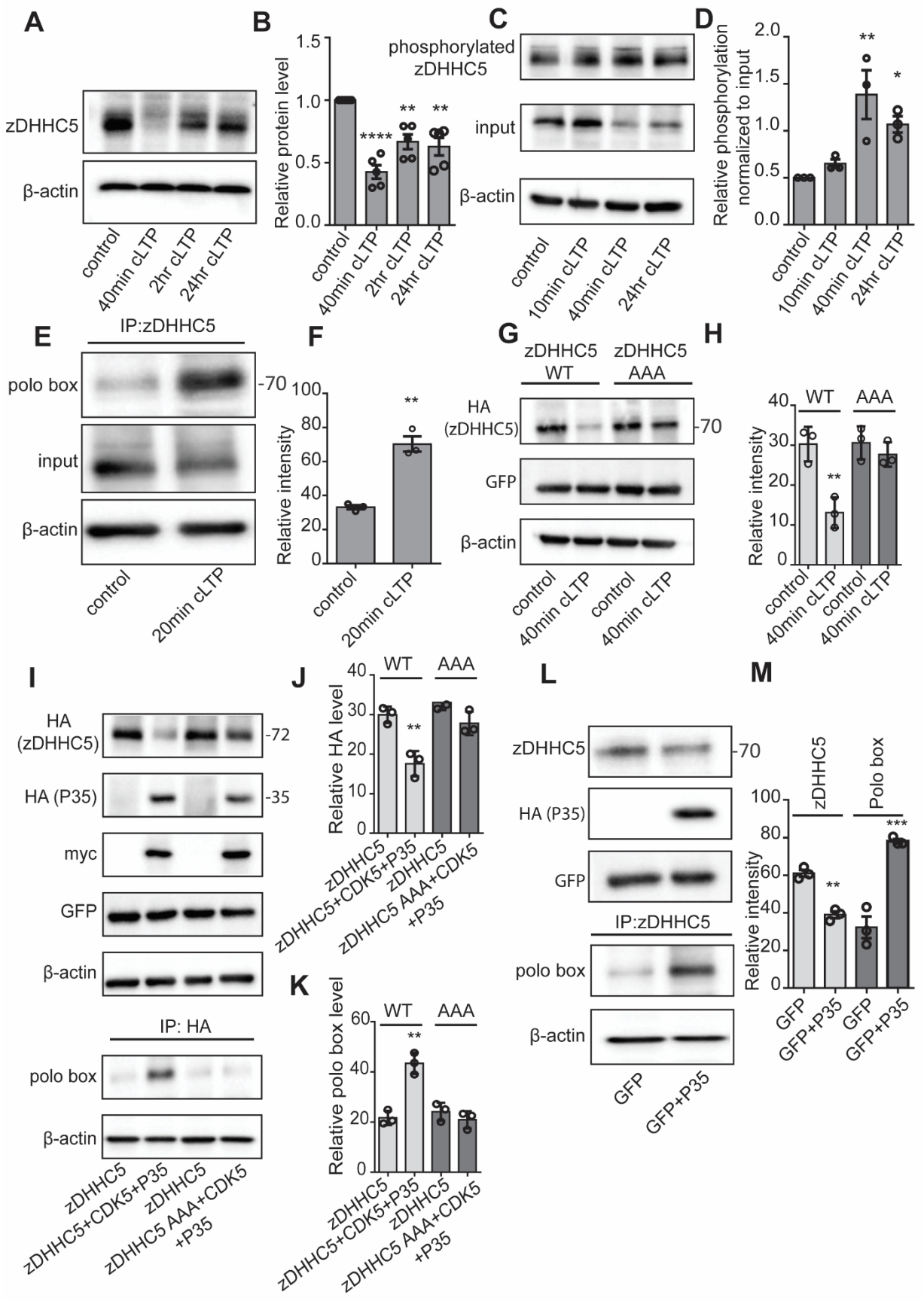
Previous work from our lab has shown that activity does not impact the palmitoylation of zDHHC5 (Brigidi et al., 2014). It was also demonstrated that zDHHC5 can differentially palmitoylate its downstream target, δ-catenin in response to increased synaptic activity by cLTP (Brigidi et al., 2015). This activity-induced palmitoylation of zDHHC5 substrate is mediated by decreased phosphorylation of Tyr533 residue and the subsequent trafficking of zDHHC5 from the synaptic membrane into dendritic shafts (Brigidi et al., 2015). While this work specifically looked at the activity-dependent phosphorylation of the particular tyrosine residue, it was next investigated if cLTP has any impact on the overall phosphorylation levels of the protein. Interestingly, a significant increase (when normalized to the input levels) in zDHHC5 overall phosphorylation levels was observed (Fig.3.4 C, D). Due to the nature of the assay used in this study to assess the protein phosphorylation (see method), the observed increase in phosphorylation could be due to changes in phosphorylation status of any phosphorylatable residue within the protein. Next, the effects of increased phosphorylation on protein stability

were examined. Notably, a significant decrease in zDHHC5 protein levels 40 minutes after cLTP treatment was observed (Fig. 3.4 A, B). Previous work has described a consensus sequence (DpSGΨXpS/T; Ψ=hydrophobic residue, X= any residue, pS or pS/T= phosphoserine or threonine) which when dually phosphorylated on serine/threonine residues, targets the protein for rapid ubiquitination and degradation (Ang et al., 2008; Arai et al., 2008; Moshe et al., 2004; Pak and Sheng, 2003; Seeburg et al., 2008). As this motif can be recognized by the 'Polo domain' present in polo-like kinases it is typically termed the Polo-box motif (Nakajima et al., 2003). As zDHHC5 has a putative Polo-box sequence (DSGIQSTP), it was first determined if increased phosphorylation of zDHHC5 occurs on Ser/Thr residues in polo box motif and whether this phosphorylation is required for activity-induced instability of zDHHC5.

20 minutes following cLTP treatment, hippocampal culture lysates were immunoprecipitated with zDHHC5 and blots probed with an antibody that specifically recognizes the phosphorylated Polo box motif (Baehr et al., 2016; Wang et al., 2018). There was a significant increase in the phosphorylation of this motif despite a significant decrease in zDHHC5 levels in cells treated with cLTP (Fig. 3.4 E, F). To determine whether the phosphorylation of this motif is required for activity-induced degradation of zDHHC5, hippocampal neurons were transfected with zDHHC5 shRNA to knockdown endogenous zDHHC5 (Brigidi et al., 2014) plus either wildtype zDHHC5 (WT zDHHC5) or phospho-mutant zDHHC5 (zDHHC5 AAA), where Ser569 and Ser572 and Thr574 in the Polo box motif were mutated to alanines. While cLTP significantly decreased the expression of wildtype HA-tagged zDHHC5, HA-tagged zDHHC5 AAA mutant levels were unchanged (Fig. 3.4 G, H), demonstrating that the phosphorylation of this motif is required for degradation of the protein.

Previous studies have shown that Polo-Like Kinase2 (PLK2) can phosphorylate residues within Polo-box motifs (Ang et al., 2008; Lee et al., 2011). We therefore determined whether PLK2 is involved in phosphorylation-dependent degradation of zDHHC5. While overexpression of WT PLK2 resulted in a decrease of WT zDHHC5, WT PLK2 has no effect on zDHHC5 AAA levels. Moreover, the PLK2 kinase-dead (KD) mutant (K108M) did not impact WT zDHHC5 levels (Fig. 3.4 N, O). Together, these results demonstrate that PLK2 mediates zDHHC5 degradation through phosphorylation of the Polo-box motif.

Prior to phosphorylation by PLK2, DpSGΨXpS/T containing peptides have shown to be phosphorylated by proline-directed kinases such as cyclin dependent kinases (CDKs) (Hamanaka et al., 1995; Martin and Strebhardt, 2006; Seeburg et al., 2008; Thomas et al., 2016). Indeed, it is likely that CDK-mediated phosphorylation can prime proteins to be phosphorylated by PLK2 (Elia et al., 2003). Previous work has identified CDK5 as the priming kinase that phosphorylates STP motifs in the substrate protein, SPAR (Seeburg et al., 2008). To see whether CDK5 is involved in the phosphorylation and destabilization of zDHHC5, hippocampal neurons were transfected with WT zDHHC5 or zDHHC5 AAA mutant together with CDK5 and its neuronal-specific activator, P35 (Chae et al., 1997). Overexpression of CDK5 and P35 increased the phosphorylation and decreased overall levels of wildtype zDHHC5 but not zDHHC5 AAA (Fig. 3.4 I- K). Endogenous CDK5 also increased the phosphorylation of the Polo-box motif and decreased stability as observed by overexpression of P35 alone (Fig. 3.4 L, M).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control
10min cLTP
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24hr cLTP
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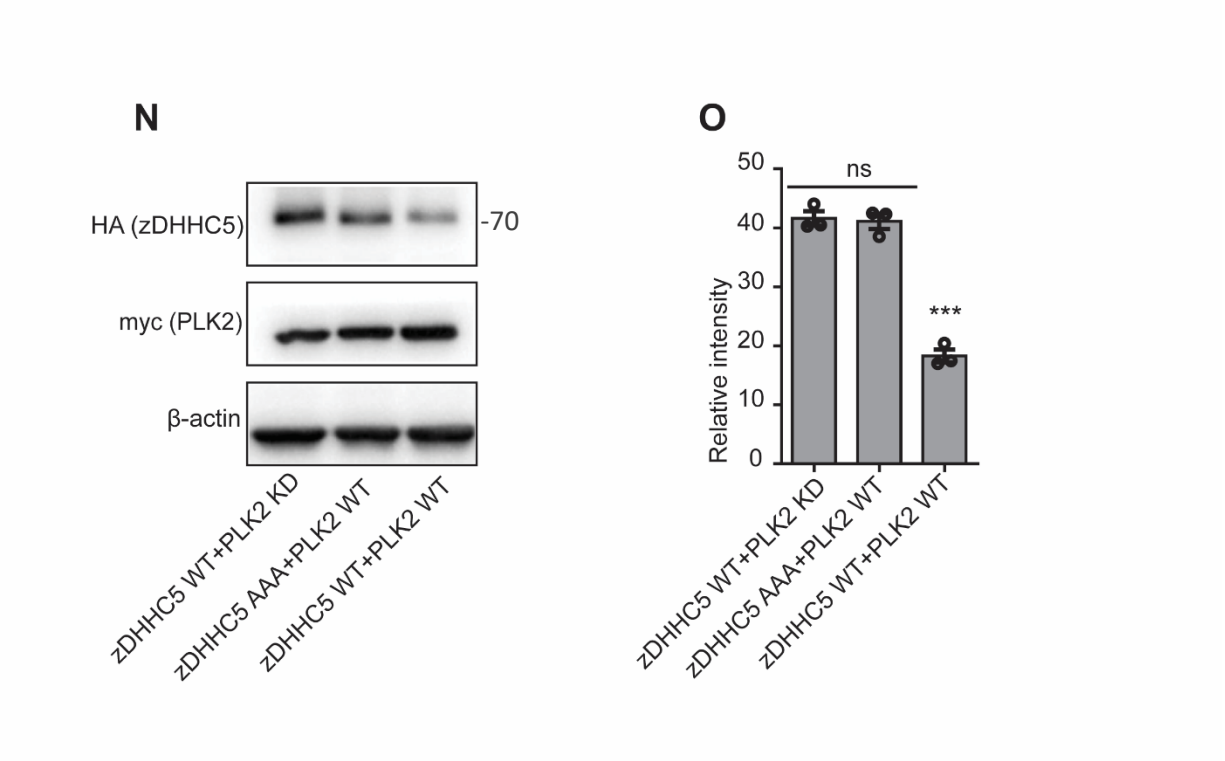

Figure 3.4. cLTP decreases zDHHC5 stability through phosphorylation of its Polo box motif

(A) Western blot analysis of changes in zDHHC5 protein levels in 14-15 DIV cultured hippocampal neurons after chemically induction of LTP at different time points. **(B)** Bar graph represents changes in zDHHC5 protein levels following increase in synapse activity relative to untreated control (N=5 separate blots from 5 separate cultures). **(C)** Phospho-protein purification assay from 14-15 DIV cultured hippocampal neurons. Phosphorylated proteins were separated by SDS-PAGE, and blots were probed with zDHHC5 antibody. As shown in panel **(D)** when normalized to the input levels, the overall phosphorylation of zDHHC5 increased 40 minutes and 24 hours after cLTP induction (N=3, separate blots from 3 separate cultures).Values are means ±SEM, *P<0.05, **P<0.01, ***P<0.001, One-way ANOVA followed by Dunnett's multiple comparisons. **(E, F)** Increased synaptic activity enhanced phosphorylation of Polo-box motif of zDHHC5 protein. N=3 separate cultures. Values are means ±SEM, *P<0.05, **P<0.01, ***P<0.001, t-test. **(G, H)** Phosphorylation of Polo-box motif is required for activity-induced degradation of zDHHC5 protein. Transfection of primary cultured hippocampal neurons by nucleofection with plasmids encoding either GFP and HA-tagged WT zDHHC5 or GFP together with HA-tagged zDHHC5 AAA mutant. The protein levels of exogenous zDHHC5 were assessed by western blot analysis of protein extracted from 14-15 DIV nucleofected neurons 40 minutes after cLTP induction, using HA antibody. N=3 separate cultures. Values are means ±SEM, *P<0.05, **P<0.01, ***P<0.001, One-way ANOVA followed by t-test. **(I-K)** Active CDK5 enhanced phosphorylation of Polo-box motif and promoted zDHHC5 degradation. 14-15 DIV cultured hippocampal neurons nucleofected with plasmids encoding: either HA-tagged zDHHC5 WT or HA-tagged zDHHC5 AAA mutant together with GFP, Myc-tagged CDK5, and its neuronal-specific activator, HA-tagged P35, were lysed and the extracted proteins were subjected to western blot analysis. N=3 separate cultures. Values are means ±SEM, *P<0.05, **P<0.01, ***P<0.001, t-test. **(L, M)** Endogenous CDK5 activity enhanced phosphorylation of Polo-box motif and promoted zdHHC5 degradation. 14-15 DIV Cultured neurons were nucleofected with either GFP alone or GFP together with CDK5 activator, P35, and total cell lysates

Figure 3.4. continued:

were immunoblotted with indicated antibodies. The total levels of Polo-box motif phosphorylation and zDHHC5 protein were normalized to control GFP-transfected cells. N=3 separate cultures. Values are means ±SEM, *P<0.05, **P<0.01, ***P<0.001, t-test. **(N, O)** PLK2-dependent degradation of zDHHC5. 14-15 DIV cultured hippocampal neurons were nucleofected with i) WT HA-tagged zDHHC5 and Myc-tagged Plk2 kinase-dead (KD) mutant, ii) HA-tagged zDHHC5 AAA and WT Myc-tagged Plk2, and iii) WT HA-tagged zDHHC5 and WT Myc-tagged Plk2. Total cell lysates were immunoblotted with indicated antibodies. N=3 separate cultures. Values are means ±SEM, *P<0.05, **P<0.01, ***P<0.001, One-way ANOVA followed by Tukey's multiple comparisons.

3.4 Synaptic activity dramatically increased zDHHC6 protein stability

zDHHC6 is a palmitoylating enzyme that, to our knowledge, has not been studied in the context of neuronal development and synaptic plasticity. Since we were able to validate an antibody that recognized the endogenous levels of the zDHHC6 protein in neurons, we next sought to determine if synaptic plasticity impacts post translational modifications or stability of the protein in neurons.

An increase was observed in zDHHC6 palmitoylation, 40 minutes post-cLTP induction that persisted even 24 hours after cLTP treatment (Fig. 3.5 C, D). As palmitoylation of zDHHC6 is known to be important for the regulation of zDHHC6 protein stability (Abrami et al., 2017), zDHHC6 protein levels were investigated following cLTP. Interestingly, a dramatic increase in zDHHC6 protein levels was observed 24 hours after cLTP induction (Fig 3.5 A, B). Changes in phosphorylation of zDHHC6 were next investigated. Although the total amount of phosphorylated zDHHC6 increased 24 hours after cLTP treatment, the magnitude of change was less than the increase in total levels of zDHHC6 protein (Fig 3.5 E). By normalizing the phosphorylated levels of zDHHC6 to the increased input levels, a relative decrease in the overall phosphorylation levels of zDHHC6 was observed 24 hours after cLTP induction (Fig 3.5 F).

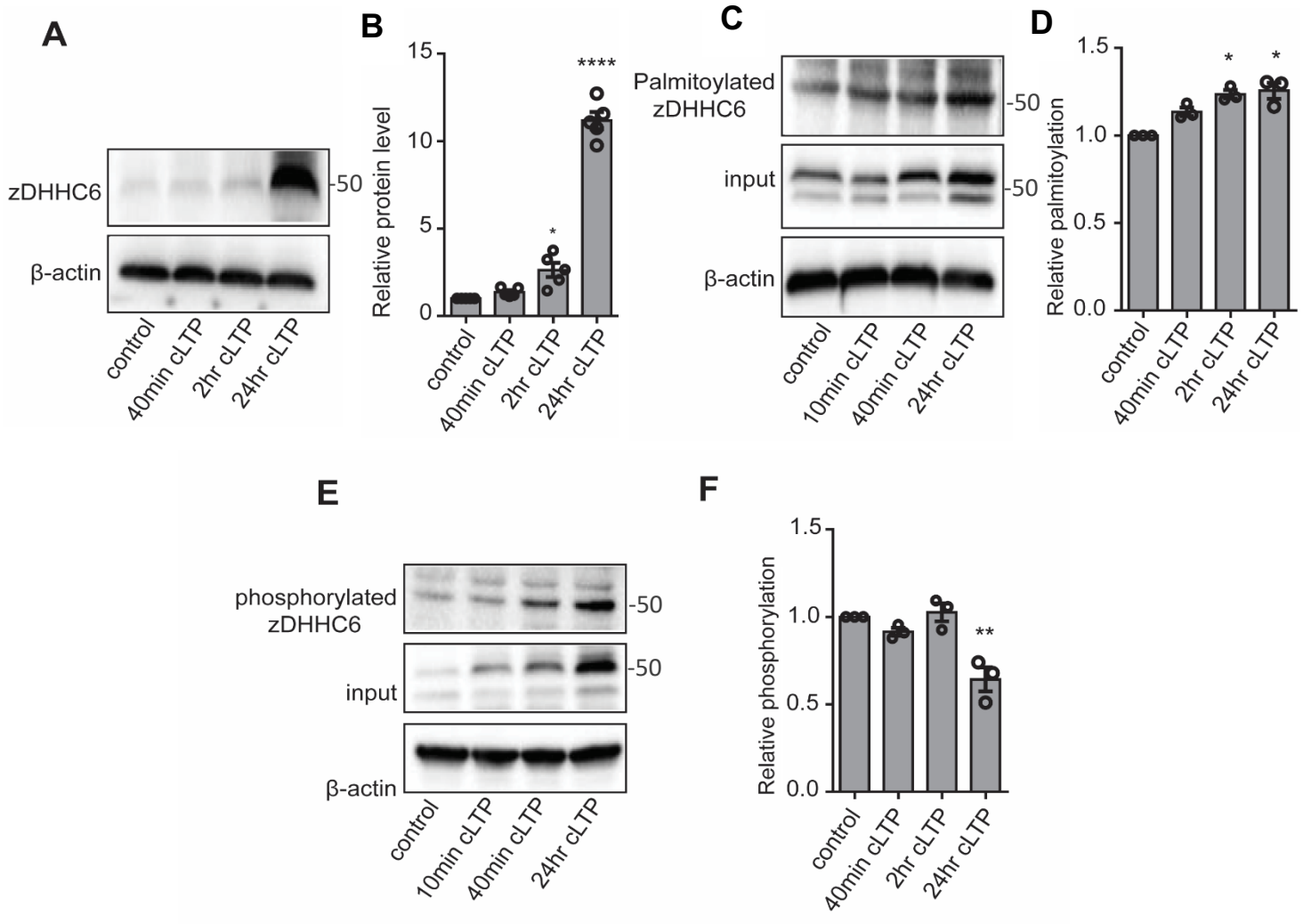

Figure 3.5. Synaptic activity increases zDHHC6 stability

(A) Western blot analysis of changes in zDHHC6 protein levels in 14-15 DIV cultured hippocampal neurons after chemically induction of LTP at different time points. **(B)** Bar graph represents changes in zDHHC6 protein levels following increase in synapse activity relative to untreated control. Increased synaptic activity dramatically increases total levels of zDHHC6 protein (N=5 separate blots from 5 separate cultures). **(C)** Acyl-Rac assay from 14-15 DIV cultured hippocampal neurons. Palmitoylated proteins were purified using Acyl-Rac assay, and were subjected to SDS-PAGE followed by blotting with zDHHC6 antibody. Increased synaptic activity resulted in an increase in total palmitoylation levels of zDHHC6. **(D)** Graph represents changes in palmitoylation of zDHHC6 protein following cLTP relative to control condition (N=3, separate blots from 3 separate cultures). All values are normalized to zDHHC6 input fraction **(E)** Phospho-protein purification assay from 14-15 DIV cultured hippocampal neurons. Phosphorylated proteins were separated by SDS-PAGE, and blots were probed with zDHHC6 antibody. As shown in panel **(F)** Overall phosphorylation levels of zDHHC6 declined 24 hours after cLTP induction (N=3, separate blots from 3 separate cultures). All values are normalized to zDHHC6 input fraction. Values are means ±SEM, *P<0.05, **P<0.01, ***P<0.001, One-way ANOVA followed by Dunnett's multiple comparisons.

3.5 cLTP does not impact stability and post-translational modifications of zDHHC8 but decreases zDHHC9 palmitoylation and its enzymatic activity

Several DHHC proteins are implicated in neurological and neurodegenerative disease (Cho and Park, 2016; Mansouri et al., 2005; Mukai et al., 2004; Raymond et al., 2007; Yanai et al., 2006). Mutation in zDHHC8, a close homologue of zDHHC5, is observed in a large population of patient with schizophrenia (Faul et al., 2005; Mukai et al., 2004). First, we sought to determine whether synaptic activity alters post-translational modifications (palmitoylation and phosphorylation) of zDHHC8 protein. In order to determine whether cLTP can affect zDHHC8 palmitoylation, palmitoylated proteins extracted from 14-15 DIV cultured hippocampal neurons were purified and subjected to SDS-PAGE. No changes were found in zDHHC8 palmitoylation 10 minutes, 40 minutes and 24 hours after cLTP treatment (Fig. 3.6 C, D). Furthermore, we did not find any changes in total levels of zDHHC8 phosphorylation following the indicated time points after Gly treatment (Fig. 3.6 E, F). Since a significant reduction in zDHHC8 mRNA levels was observed 24 hours after cLTP (Fig. 3.1 A), it was investigated if changes in mRNA levels translate into changes in the protein levels of zDHHC8. Unlike the significant reduction in zDHHC8 mRNA levels 24 hours after cLTP treatment, we did not see any changes in its protein levels 40 minutes, 2 hours and 24 hours after cLTP treatment (Fig. 3.6 A, B). This suggests that zDHHC8 may not be highly responsive to synaptic stimulation with cLTP.

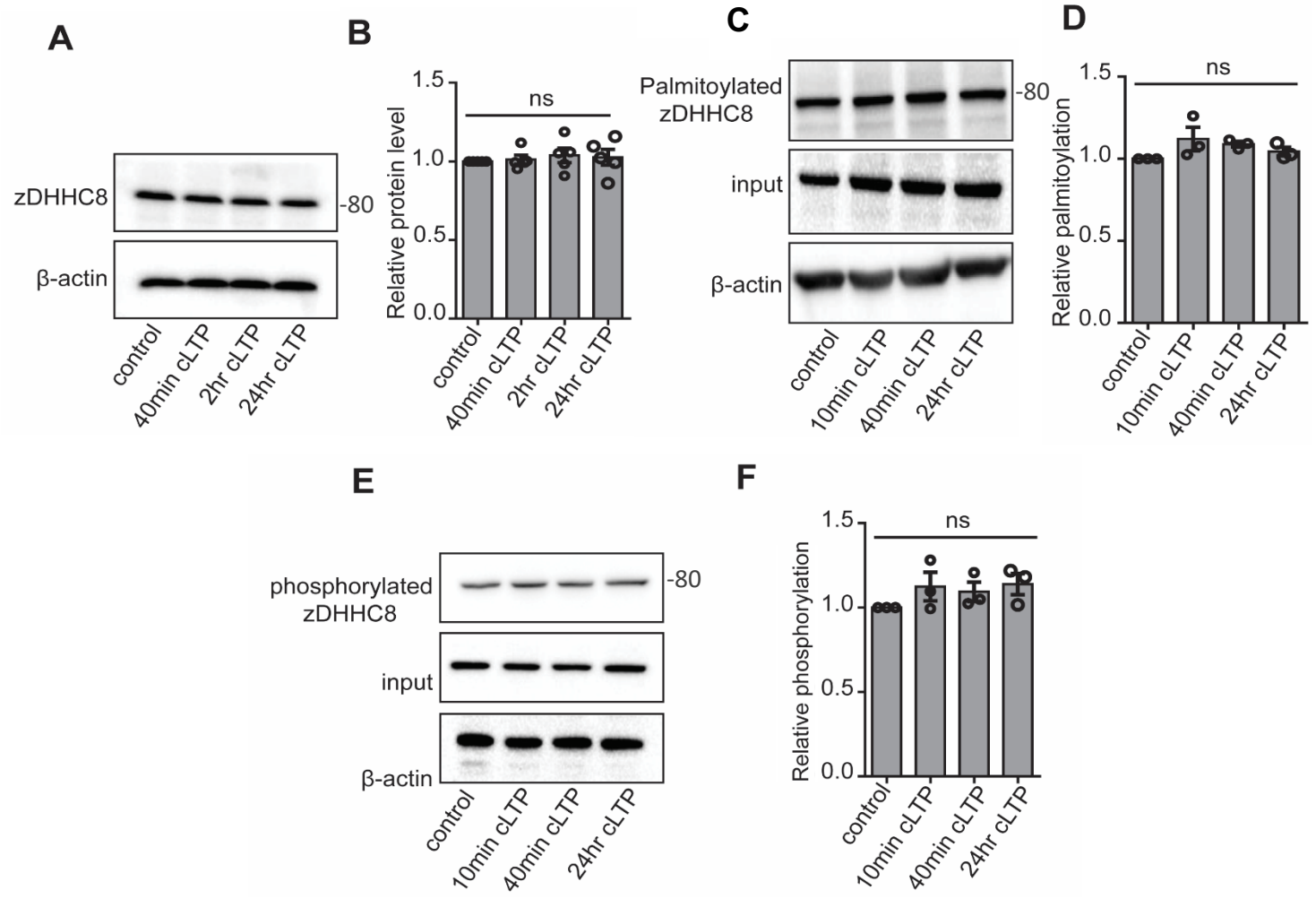

Figure 3.6. zDHHC8 protein levels and its post-translational modifications were unchanged following cLTP treatment

(**A, B**) There is no change in zDHHC8 levels 40 minutes, 2 hours and 24 hours after cLTP treatment in 14-15 DIV hippocampal cultures. (N=5 separate blots from 5 separate cultures). (**C, D**) Acyl-Rac assay from 14-15 DIV cultured hippocampal neurons. Palmitoylated proteins were purified using Acyl-Rac assay, and were subjected to SDS-PAGE followed by blotting with zDHHC8 antibody. There were no changes in overall levels of palmitoylated zDHHC8 protein following cLTP treatment. (**D**) Graph represents changes in palmitoylation of zDHHC8 protein following cLTP treatments relative to control condition. All values are normalized to zDHHC8 input fraction (N=3, separate blots from 3 separate cultures). (**E**) Phospho-protein purification assay from 14-15 DIV cultured hippocampal neurons. Phosphorylated proteins were separated by SDS-PAGE, and blots were probed with zDHHC8 antibody. Increased synapse activity did not impact overall phosphorylation levels of zDHHC8. (**F**) statistical evaluation of changes in overall levels of zDHHC8 phosphorylation following increased synapse activity. All values are normalized to zDHHC8 input fraction (N=3, separate blots from 3 separate cultures). Values are means ±SEM, *P<0.05, **P<0.01, ***P<0.001, One-way ANOVA followed by Dunnett's multiple comparisons.

zDHHC9 has also been shown to be involved in intellectual disabilities (Mitchell et al., 2014; Raymond et al., 2007). Our lab recently demonstrated the important role of zDHHC9 protein in the regulation of dendritic outgrowth and excitatory-inhibitory balance through promoting inhibitory synapse formation (Shimell et al., 2019). Due to the importance of zDHHC9 in the regulation of synapse formation, it was investigated whether zDHHC9 is impacted by synapse activity.

In order to determine if synaptic stimulation with cLTP can impact zDHHC9 phosphorylation, phosphorylated proteins were purified using PhosphoProtein purification assay from 14-15 DIV cultured hippocampal neurons. Following increased synaptic activity by cLTP overall zDHHC9 phosphorylation levels were unchanged (Fig. 3.7 G, H). However, the overall palmitoylation levels of zDHHC9 significantly decreased 10 minutes after cLTP treatment. Indeed, zDHHC9 palmitoylation decreased by half 10 minutes after cLTP and was maintained at this low level even 24 hours after cLTP treatment (Fig. 3.7 C, D).

Recent work from our lab has identified TC10 as a novel substrate for zDHHC9 (Shimell et al., 2019). To determine whether the decrease in zDHHC9 palmitoylation translates into changes in its enzymatic activity, the palmitoylation of its known substrate, N-Ras, and its novel substrate, TC10, were tested 1 hour after cLTP treatment. Interestingly, a significant decrease in the palmitoylation of these two proteins was observed (Fig. 3.7 E, F), indicating that synaptic stimulation with cLTP impacts palmitoylation of zDHHC9 which in turn results in changes in the enzymatic activity of the enzyme. Indeed, this demonstrates an interesting example of how synaptic activity can regulate the dynamic palmitoylation of substrates by affecting post translational modification of the enzymes responsible for palmitoylation.

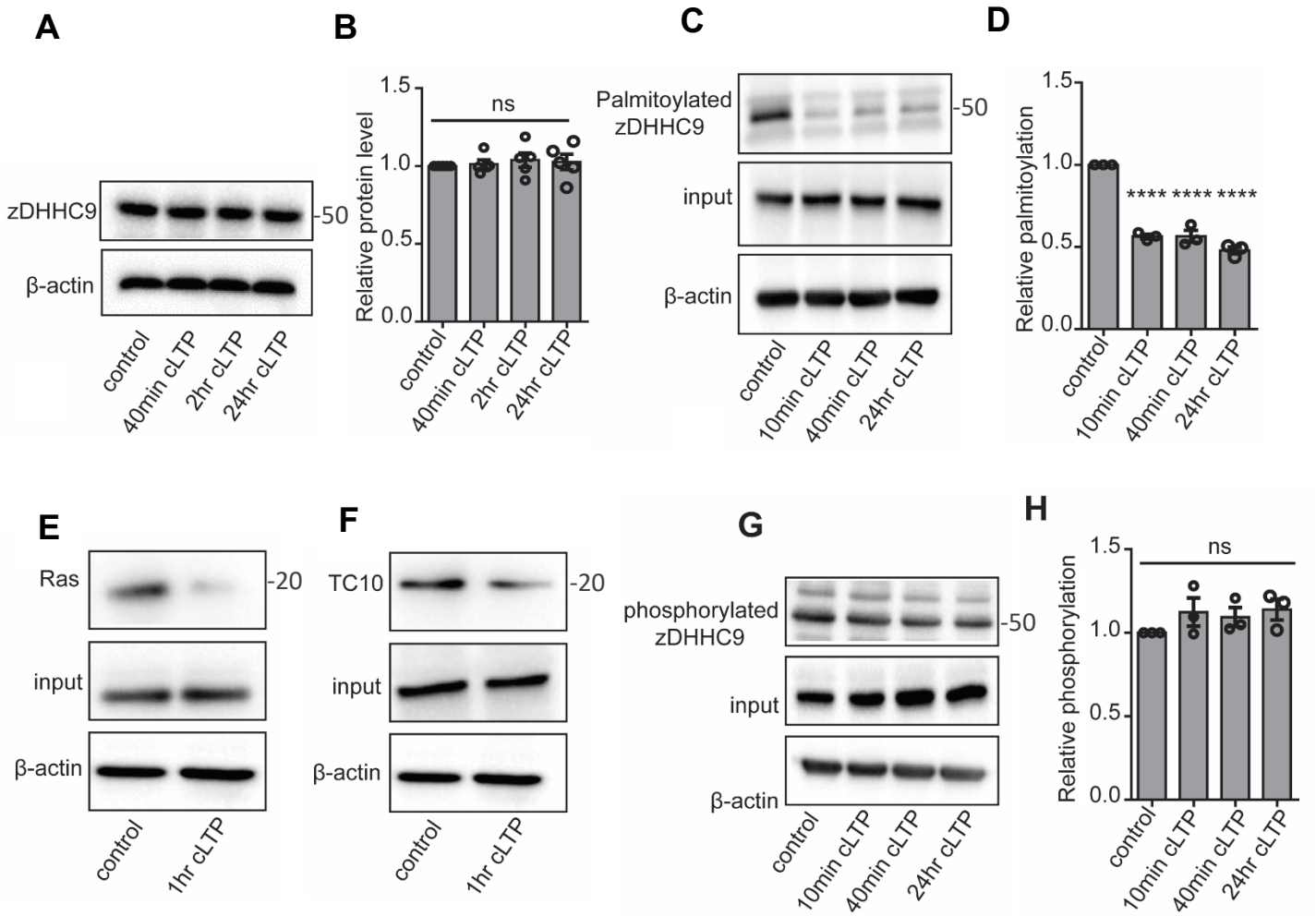

Figure 3.7. Activity decreases zDHHC9 palmitoylation and its enzymatic activity

(A) Western blot analysis of changes in zDHHC9 protein levels in 14-15 DIV cultured hippocampal neurons after chemically induction of LTP at different time points. **(B)** Statistical evaluation of changes in zDHHC9 protein levels following increase in synapse activity relative to untreated control. Increased synaptic activity did not alter total levels of zDHHC9 protein (N=5 separate blots from 5 separate cultures). **(C)** Acyl-Rac assay from 14-15 DIV cultured hippocampal neurons. Palmitoylated proteins were purified using Acyl-Rac assay, and were subjected to SDS-PAGE followed by blotting with zDHHC9 antibody. (**D**) Graph represents changes in palmitoylation of zDHHC9 protein following cLTP treatments relative to control condition. Increased synaptic activity resulted in a significant decrease in the levels of zDHHC9 palmitoylation. All values are normalized to zDHHC9 input fraction (N=3, separate blots from 3 separate cultures). **(E, F)** Decrease in palmitoylation of zDHHC9 substrates, Ras and TC10, 1 hour following cLTP treatment, N=1. **(G)** phospho-protein purification assay from 14-15 DIV cultured hippocampal neurons. Phosphorylated proteins were separated by SDS-PAGE, and blots were probed with zDHHC9 antibody. Increased synapse activity did not impact overall phosphorylation levels of zDHHC9. **(H)** statistical evaluation of changes in total levels of zDHHC9 phosphorylation following increased synapse activity. All values are normalized to zDHHC9 input fraction (N=3, separate blots from 3 separate cultures). Values are means ±SEM, *P<0.05, **P<0.01, ***P<0.001, One-way ANOVA followed by Dunnett's multiple comparisons.

Chapter 4: Discussion

Previous studies identified a number of critical synaptic proteins such as PSD-95 (El-Husseini et al., 2002a; Noritake et al., 2009), δ-catenin (Brigidi et al., 2014), and AKAP79/150 (Keith et al., 2012) that are differentially palmitoylated following synaptic activity. This activity-regulated palmitoylation mediates trafficking and localization of synaptic substrates, and thus modulates synaptic plasticity and function. Despite the important role of this modification, the molecular mechanisms underpinning activity-mediated palmitoylation of synaptic proteins is largely unknown.

In the present study, post-translational modifications to the enzymes responsible for palmitoylation are reported in response to synaptic stimulation with cLTP, which in turn impact the stability as well as enzymatic activity of the enzymes. Notably, although transcriptional profiles of PATs might not be largely impacted by synaptic activity, post translational modifications of four PATs (out of five studied enzymes) are highly regulated by synaptic stimulation with cLTP. Moreover, no changes were observed in transcriptional profiles nor post translational modifications of thioesterase, ABHD17. Together, while the main objective of this work was to elucidate *how* activity-induced differential palmitoylation of synaptic substrates takes place, the data presented herein suggest that this process is more likely through activity-mediated changes occurring in palmitoylating enzymes. These dynamic changes in the enzymes are thought to be important for downstream palmitoylation of synaptic proteins and the modulation of synapse plasticity.

4.1 cLTP does not largely impact transcriptional profiles of zDHHC and ABHD17 enzymes

The influx of Ca^{2+} through NMDARs during synapse activity has shown to induce expression of a number of genes which themselves in turn contribute to different phases of long-term synaptic plasticity (West and Greenberg, 2011). In fact, based on differential screens for activity-regulated genes, and among thousands of genes expressed in the nervous system, approximately 300 genes are predicted to be regulated by activity (Hevroni et al., 1998; Nedivi, 1999). This activity-mediated gene expression is thought to be critical for the formation and maintenance of long-term synaptic changes. Based on the timescale over which synaptic change (here LTP) persists, this phenomenon can be divided into at least two distinct phases: an early phase (E-LTP), and a late phase (L-LTP). E-LTP is largely mediated by post-translational modifications and regulation of existing proteins, and thus is independent of new protein synthesis. In contrast, for L-LTP to occur, transcription factor activation, new gene transcription, and protein synthesis are required (Baltaci et al., 2019).

Our results from the expression profiles of zDHHCs and ABHD17s demonstrate that the transcription of these enzymes is not mainly affected by synaptic stimulation with cLTP and thus the product of these genes in long-term may not be involved in the maintenance of long-lasting forms of synaptic plasticity. However, post-translational modifications occurring on zDHHCs following synaptic stimulation with cLTP may suggest a potential role for these enzymes in the regulation of early phase of LTP. Although decreased mRNA levels were observed for some zDHHCs (zDHHC2, and 8), those transcriptional changes did not appear to translate into changes in their protein levels. Unfortunately, due to the lack of specific antibodies for the other

two zDHHCs with altered mRNA levels (zDHHC11, and 22) we were not able to conclude if transcriptional changes necessarily lead to changes in their protein levels.

4.2 Synaptic activity impacts zDHHC2 phosphorylation

A number of critical synaptic proteins including PSD-95 (Fukata et al., 2004a), AKAP79/150 (Woolfrey et al., 2015), and the SNAP25 (Greaves et al., 2010) protein family have shown to be palmitoylated by zDHHC2. Apart from these incredibly important synaptic substrates, dynamic trafficking, as well as the synaptic localization of zDHHC2, have made this enzyme one of the most attractive PATs to study in the context of synapse biology.

The postsynaptic scaffolding protein, PSD-95, represents a major palmitoylated protein in the brain (Topinka and Bredt, 1998). Indeed, the palmitoylation of PSD-95 has shown to be critical for its synaptic trafficking and thereby recruitment and clustering of AMPAR subunits into the synapses (El-Husseini et al., 2000). Interestingly, PSD-95 palmitoylation has been shown to be a dynamic process, regulated by changes in synaptic activity (El-Husseini et al., 2002b). This dynamic palmitoylation is achieved by rapid trafficking of zDHHC2 to synaptic membranes, where it can palmitoylate its substrate PSD-95, and has shown to be important for homeostatic regulation of neuronal activity (Noritake et al., 2009). Complementing these findings, a recent study indicated that the translocation of zDHHC2 is regulated by its C-terminal domain. In fact, potential phosphorylation of two distinct sites (SQ, and NP motifs) within the C-terminal domain of zDHHC2 has been suggested to affect its localization (Salaun et al., 2017). Notably, the activity-induced phosphorylation of one of these sites (SQ motif) is supported by another study looking at the phosphorylation of SQ-containing substrates in response to changes

in synaptic activity (Siddoway et al., 2014). These findings highlight a potential role for phosphorylation in modulation of zDHHC2 trafficking as well as its localization, and are to a degree consistent with our observation of an activity-dependent overall changes in phosphorylation of zDHHC2. However, whether the phosphorylation of the C-terminus of the protein, in particular is impacted by enhanced synaptic activity remains unknown. It is worthwhile to examine whether activity-induced changes in phosphorylation of zDHHC2 affect the trafficking and colocalization or interaction of the enzyme with its synaptic substrates, PSD-95 and/or AKAP79/150.

Dynamic palmitoylation of PSD-95 also requires the 'removal' of palmitate group, and thus completing a cycle of this post translational modification. Recently, ABHD17A, 17B, and 17C have been identified as depalmitoylating enzymes for PSD-95 in neurons (Lin and Conibear, 2015; Yokoi et al., 2016). It is possible that activity-dependent palmitoylation of PSD-95 is regulated through changes in ABHD17s. Our qPCR data demonstrates that enhanced synapse activity does not alter transcription of ABHD17 enzymes. Overexpressing all three ABHD17 proteins in HEK cells demonstrated that our antibody only recognized ABHD17B, which in fact has shown to have the greatest depalmitoylation effect on PSD-95 (Yokoi et al., 2016). Here, no changes were observed in protein levels, nor post-translational palmitoylation/ phosphorylation of this enzyme following cLTP, suggesting the possibility that the activity-induced palmitoylation of PSD-95 is regulated by its palmitoylating enzyme, and less likely through thioesterase ABHD17.

4.3 Synaptic activity reduces zDHHC5 stability

The ubiquitous expression of zDHHC5 in the brain, as well as its implication in brain disorders such as schizophrenia (Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014) and bipolar disorders (Fallin et al., 2004) highlight an essential role for zDHHC5 in neuronal regulation. In fact, dynamic palmitoylation of δ-catenin by zDHHC5 has recently been shown to play an important role in regulation of synapse plasticity (Brigidi et al., 2014). This dynamic palmitoylation has shown to occur through activity-induced changes in the subcellular trafficking of zDHHC5 (Brigidi et al., 2015). Under basal conditions, zDHHC5 was shown to be localized to the synaptic membranes. However, increasing neuronal activity with cLTP leads to its translocation to dendritic shafts where it can palmitoylate its substrate, δ-catenin. Palmitoylated δ-catenin subsequently stabilizes N-cadherin, which in turn results in the cadherin-mediated AMPAR stabilization. Indeed, this activity-induced trafficking of zDHHC5 highlights the enzyme responsivity to changes in synapse efficacy. In accordance with this, we also observed a rapid change in the total protein levels of zDHHC5 following enhanced synaptic activity. Interestingly, changes in zDHHC5 protein levels have been previously reported in response to differentiation signals (Li et al., 2012). While those signals are not the same as cLTP, it is prior evidence that rapid changes in zDHHC5 and subsequent changes in the palmitoylation of zDHHC5 substrates can have impact on cellular phenotypes.

zDHHC5 was also reported to palmitoylate GRIP1b, a glutamate receptor interacting protein. GRIP1b has shown to be localized to postsynaptic spines and its palmitoylation by zDHHC5 was shown to increase AMPAR turnover (Thomas et al., 2012). It is, therefore, interesting to speculate that enhanced synaptic activity leads to stabilization of AMPARs through affecting zDHHC5 stability and trafficking. Indeed, it is possible that reduced levels of zDHHC5

following synapse activity, in addition to its trafficking to the shaft enhance AMPAR stabilization in two ways; while the former could result in a decrease in the palmitoylation of synaptically localized GRIP1b, and therefore, a possible reduction in AMPAR turnover, the later leads to an increase in palmitoylation of δ-catenin and thus cadherin-mediated AMPAR stabilization and strengthening of the synapses.

4.4 Synaptic activity increases zDHHC6 stability

zDHHC6 is known to palmitoylate key proteins such as ER chaperone calnexin (Lakkaraju et al., 2012), IP_3 receptor (Fredericks et al., 2014), and transferrin receptor (Senyilmaz et al., 2015). For each of these proteins, palmitoylation was shown to regulate stability, localization, trafficking and function. Despite the ever-increasing roles and novel targets of palmitoylation, little is known about the dynamics and regulation of the mediating enzymes themselves.

In this study, zDHHC6 protein stability dramatically increased following synaptic stimulation with cLTP. The stability of a number of proteins has previously been shown to be affected by post-translational palmitoylation (Dallavilla et al., 2016; Fairbank et al., 2012; Linder and Deschenes, 2007; Rossin et al., 2015). zDHHC6 is known to be palmitoylated on three cysteine residues (Collins et al., 2017). Interestingly, previous work has demonstrated that palmitoylation of a particular cysteine residue (Cys-329) within the C-terminal domain of the protein can have a stabilizing effect, increasing the half-life of the protein up to 100 hours (Abrami et al., 2017). Consistent with this observation, we report here an increase in zDHHC6 palmitoylation following cLTP. It is therefore tempting to speculate that increased synaptic

activity increases the stability of the protein through palmitoylation of that particular site in the protein. Further work is required to study this in detail.

zDHHC6 is in fact the first PAT known to be palmitoylated by an upstream palmitoyltransferase, zDHHC16, revealing, for the first time, that the formation of palmitoyl-intermediate is not necessarily through autoplamitoylation of the PATs (Abrami et al., 2017). As zDHHC6 can be palmitoylated by an upstream enzyme, zDHHC16 it would be worthwhile to examine if cLTP affects the enzymatic activity of zDHHC16, or its interaction with its substrate, zDHHC6.

Together, although the potential synaptic substrates involved in the regulation of synaptic activity for zDHHC6 have not been identified yet, the observation that many aspects of the enzyme, such as its stability, palmitoylation, and phosphorylation are impacted by synaptic activity might suggest a potential role for this enzyme in the modulation of synaptic plasticity. Further analysis is required to identify the potential substrates.

4.5 Synaptic activity reduces zDHHC9 enzymatic activity

zDHHC9 is one of the first identified PATs in yeast (Lobo et al., 2002), and is known to palmitoylate important proteins in cellular processes. Here, an activity-driven reduction in post-translational palmitoylation of zDHHC9 was observed. Interestingly, this reduction is also observed in palmitoylation of zDHHC9 substrates. This raises the possibility that neuronal activity can regulate palmitoylation of substrates through affecting PATs. In the case of zDHHC9, this regulation may take place through changes in enzymatic activity. Two possibilities likely underlie the activity-mediated changes in zDHHC9 enzymatic activity. The

decrease in palmitoylation of cysteine residue within the DHHC motif is one possibility. The palmitoylation of cysteine residue in this motif has shown to be important for the formation of palmitoyl-intermediate and, therefore, enzymatic activity of the PATs (Roth et al., 2002). It is also possible that synaptic activity impacts the association of zDHHC9 with its cofactor, GCP16. This interaction has shown to be required for proper enzymatic activity of the enzyme (Swarthout et al., 2005) which is likely through increasing protein stability as well as stabilizing the palmitoyl-intermediate.

In addition to its physiological roles, zDHHC9 has shown to be implicated in X-linked intellectual disability (Masurel-Paulet et al., 2014; Mitchell et al., 2014; Raymond et al., 2007). Palmitoylation of neuronal proteins by zDHHC9 is, therefore, likely essential for normal neuronal function. A recent work revealed a potential role for zDHHC9 in the regulation of neuronal connectivity (Shimell et al., 2019). It is shown that while zDHHC9 promotes dendritic outgrowth through palmitoylation of Ras, it can promote inhibitory synapse formation by palmitoylation of its novel substrate, TC10. It is therefore possible that the decrease in palmitoylation of Ras and TC10 results in fewer inhibitory synapses, and shorter dendritic length, which both can potentially lead to an overall increase in network connectivity.

Together, these findings identify differential regulation of DHHC-type palmitoylating enzymes in response to changes in neuronal activity and raise the possibility that these activity-dependent changes in PATs are involved in differential palmitoylation of neuronal proteins. Identification of additional mechanisms regulating the dynamic palmitoylation of synaptic substrates remains an exciting area for future investigation.

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