UNIVERSITY OF CALIFORNIA, SAN DIEGO

A plasma membrane protein proteomic approach to elucidate the role of ubiquitin-dependent endocytosis and lysosomal degradation in cortical neurons

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in

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by

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The thesis of April Baoyi Guan is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Chair

University of California, San Diego

2016

Dedication

I dedicate this thesis to everyone who gave me strength when I was weak, especially my mom, John, Chuck, Jessie and May. I couldn't do it without your support.

成功乃是失败之母。

(Failure is the mother of success.)

The Flood Control by Gun and Yu

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Acknowledgments

Thinking back, I still remember the interview talk I had with my thesis adviser, Dr. Gentry Patrick, as a freshman applying for lab assistant job in his lab. He explained what his lab does and what he expected from me. Honestly, I did not understand a word he says at that time, I was just this miserable freshman trying to figure out what I want to do. Never have I thought I would have the honor to be his student for the next five years. He provided all the resource and opportunity I needed even I was just a freshman washing dishes in his lab. When I asked for hands-on research, Dr. Gentry Patrick gave me a project without hesitation; he has trust in me more than I have trust in myself. I remember Dr. Gentry Patrick saying that research is not a nine to five job, it requires real commitment, and he has never been so right. I admit there were times I wanted to give up, thank you for pulling me back in during those times.

Not only I have gained enormous knowledge on scientific research, I have obtained lifetime friendships all because of this lab. Alice Molteni and Feline Lindhout, who I can count on anytime, every bit of moment we spent together has been a real bless. We have shared laughter, frustrations, and tears, thank you for making me a better person and giving all the supports I needed in and outside of the lab. Frankie Gonzales, who shows his affections through mockery, you have inspired me to be a stronger person. Despite all the “attacking,” I do not know what I would do without you, I have learned so much from you, thank you for being so real and kind to me.

ABSTRACT OF THE THESIS

A plasma membrane protein proteomics approach to elucidate the role of ubiquitin-dependent endocytosis and lysosomal degradation in cortical neurons

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Ubiquitin-dependent receptor endocytosis and subsequent endocytic sorting to the lysosome for degradation plays an essential role in synaptic plasticity by changing the number of membrane receptors at the synaptic sites. However, our knowledge of this pathway on the overall synaptic membrane protein population is still rudimentary. Here,

we identify surface synaptic membrane proteins as candidate ubiquitinated targets of the lysosome. We show that the lysosomal inhibition increases surface synaptic membrane protein ubiquitination in neurons. We combine the isolation of biotinylated cell surface membrane proteins, lysosomal perturbation and mass spectroscopy analysis to generate the first large-scale proteomic analysis of the surface synaptic proteome as candidate ubiquitinated targets of the lysosome. After 12 hours treatment, we compared biochemically purified synaptic cell membrane protein fractions between normal and lysosome-perturbed conditions through tandem mass spectrometry. A total of 1600 proteins were identified and quantified, including 539 plasma membrane proteins. Out of these plasma membrane proteins, we found that 105 of them were upregulated, and 207 were downregulated, while 226 did not significantly change in protein level. The proteins with altered pattern include proteins involved in regulating cell adhesion, cellular homeostasis, synaptic transmission, and cellular localization. This suggests that the ubiquitin-dependent lysosomal degradation pathway is important in maintaining synaptic function.

I. Introduction

Synaptic plasticity

Our brain is remodeled constantly in response to our daily activities such as learning and recalling past events (Morris 1999). This alternation is called synaptic plasticity, which refers to the ability of synapses to strengthen or weaken in response to the change of activities (Hughes 1958).

There are two well-known mechanisms to attain synaptic plasticity, which are to change the level of neurotransmitters such as glutamate released into a synapse, and to change the number of transmembrane receptors on the postsynaptic membrane (Schwarz and Patrick 2012, Goo, Scudder et al. 2015). The ability of a neuron to respond to outside stimuli depends on the density of transmembrane receptors at the postsynaptic density (Malinow and Malenka 2002, Malenka 2003). To regulate the density of the membrane population of these receptors, transmembrane receptors are added to the membrane by exocytosis and removed by endocytosis. Endocytosis of transmembrane receptors involves a modification step that signals them for internalization, and a delivery step in which the receptors are either sent for degradation or recycled back to the cell surface (Marsh and McMahon 1999). In recent years, studies have shown that ubiquitination acts as a signal for the internalization and sorting of plasma membrane proteins.

Ubiquitination

Ubiquitination is the covalent modification of proteins in which a highly conserved 76 amino acid protein called ubiquitin is added to a lysine residue on the target proteins, and these ubiquitinated proteins are sent for degradation (Pickart and Eddins 2004). Ubiquitination is a highly regulated process in mammalian cells. It involves three

main enzymatic steps. First, ubiquitin is activated by the ubiquitin-activating enzymes (E1s); then this activated ubiquitin is transferred to the ubiquitin-conjugating enzymes (E2s), and ligates with substrates through the help of ubiquitin ligase (E3s) (Ciechanover 1998). There are several types of ubiquitin modification: monoubiquitination, the attachment of a single ubiquitin; multiple monoubiquitination, the attachment of multiple single ubiquitin molecules to several lysine residues in the target protein, and polyubiquitination, a modification with ubiquitin chains of diverse lengths and linkages. After transmembrane proteins are ubiquitinated, they are internalized either by clathrin-dependent or clathrin-independent endocytosis mechanism. They then proceed to the delivery step, where they are either sent for degradation or recycled back to the cell surface (Sorkin and von Zastrow 2009). Depending on the topology and length of the ubiquitin chain, the fate of the ubiquitinated protein such as interaction, localization can be changed. Ubiquitination is a highly reversible process. Ubiquitinated proteins can be reversed its modification by the action of deubiquitinating enzymes (DUBs), and these de-ubiquitinated proteins will then recycle back to the cell surface.

Ubiquitin-dependent degradation pathways

There are two main degradation pathways for ubiquitinated proteins: the Ubiquitin Proteasomal System (UPS) and the lysosomal pathway (Pickart and Eddins 2004). The destined degradation pathway of ubiquitinated proteins depends on the type of ubiquitination. Proteins with a single ubiquitin molecule attached is called mono-ubiquitinated proteins; proteins with additional ubiquitin molecules attached and form a polyubiquitin chain is called polyubiquitination, which are usually targeted to the

ubiquitin-proteasomal degradation pathway and degraded by the large multi-subunit protease called the 26S proteasome. The synaptic membrane proteins are typically mono-ubiquitinated or with short chain of ubiquitin, and they are normally degraded by the lysosomal degradation pathway (Pickart and Eddins 2004, Clague and Urbe 2010). Membrane proteins are first tagged with ubiquitin, which are recognized by the endosomal sorting complexes required for transport (ESCRT) machinery and recruited to the endosome. Endosome is then developed into late endosome and becomes the multi-vesicular body (MVBs); it is fused with lysosome for protein degradation (Piper and Luzio 2007). If a DUB reverses ubiquitination of the targeted protein in the early endosome, the protein can be recycled back to the plasma membrane.

Ubiquitination of epidermal growth factor receptor (EGFR)

One well-studied example of ubiquitin-dependent endocytosis of surface receptor and subsequently targeted for lysosomal degradation is the epidermal growth factor receptor (EGFR). Extensive studies have shown that direct ubiquitination of EGFR by c-Cb1 serve as a signal for the assembly of clathrin-mediated endocytic machinery and subsequent endocytosis, and endosomal sorting for degradation (de Melker, van der Horst et al. 2001, Goh and Sorkin 2013). These studies indicate that ubiquitination acts as a necessary signal to send receptor for endosomal sorting and subsequent lysosomal degradation. They show that mutant EGFRs without ubiquitination sites are not targeted for lysosomal degradation but send back to the plasma membrane from the endosomes, this implies that ubiquitination is required for the subsequent lysosomal degradation (Raiborg, Bache et al. 2002, Peschard and Park 2003).

Ubiquitination in endocytosis and endosomal sorting of AMPARs and GABARs

Another striking example of a synaptic membrane protein that is degraded by the lysosomal degradation pathway is the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR), an ionotropic transmembrane receptor for glutamate, which mediates synaptic transmission (Schwarz and Patrick 2012, Goo, Scudder et al. 2015). AMPARs are known for their role in synaptic plasticity; by regulating the amount of neurotransmitters transported into the cell, AMPA receptors can initiate long-term potentiation (LTP) and long-term depression (LTD) (Shepherd and Huganir 2007). Therefore, changing the number of AMPA receptors on the postsynaptic membrane is one way to achieve synaptic plasticity. Recent studies found that stimulation of AMPA receptors by the agonist AMPA causes robust ubiquitination, leads to internalization, and subsequent trafficking into lysosomal degradation pathway for degradation in hippocampal neurons (Shepherd and Huganir 2007, Schwarz, Hall et al. 2010). Besides AMPAR, ubiquitin-dependent regulation and lysosomal degradation has been shown to be crucial for inhibitory synaptic transmission. γ-Aminobutyric acid (GABA) receptors, inhibitory synaptic receptors that hinder action potential, have also been found to be modified by ubiquitin and degraded by the lysosomes; mutation of their ubiquitination sites disables their ability to be degraded by the lysosomes (Arancibia-Carcamo, Yuen et al. 2009). There are many other surface proteins that are modified by ubiquitin and trafficked to the lysosomes for degradation; it is crucial to identify these components and connect them with synaptic plasticity.

The proteasome, lysosome, and autophagy

We mentioned the two main ubiquitin-dependent degradation pathways, and how their regulation can affect synaptic plasticity. Another degradation pathway, which does not involve ubiquitin modification, but also degrades damaged cytoplasmic proteins and cell organelles with the help of autophagosomes, it's the autophagy pathway (Mizushima 2007). Although this pathway does not involve in ubiquitination, it is part of the lysosome system; therefore, dysfunction of this pathway might affect the efficacy of the endosome-lysosome pathway. One study showed that lysosome is activated through a dual mechanism between mTORC1 suppression and autophagosome-lysosome fusion (Zhou, Tan et al. 2013). Since the endosome-lysosome system has been shown to play a crucial role in synaptic plasticity, the autophagy pathway could also be a player in synaptic plasticity. In fact, there are a few reports indicate this relationship (Shen and Ganetzky 2009, Shehata, Matsumura et al. 2012); the role of this pathway is still rudimentary.

Neurodegeneration involves proteolytic dysfunction

Synaptic strength is partly depended on the number of receptors at the postsynaptic surface; for example, it is shown that insertion of AMPA-type glutamate receptor (AMPARs) at the synapse lead to long-term potentiation. Therefore, looking the change of overall membrane proteins in response to lysosomal inhibition can unravel the role of lysosomal degradation pathway in synaptic plasticity. Furthermore, impairment in synaptic plasticity underlies the cause of many neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease; numerous studies have shown a strong

correlation between synapse impairment and accumulation of plaque and tangle (Hardy and Selkoe 2002, Rodrigues, Scudder et al. 2016). These studies suggest that these plaques, particularly pathogenic peptide amyloid β-protein (Aβ), hinder synaptic transmission by promoting downregulation of surface AMPARs through ubiquitination, internalization, and subsequent endocytic sorting to the lysosome for degradation (Rodrigues, Scudder et al. 2016). Observing membrane protein changes in response to lysosomal inhibition in a global scale can not only help us understanding synaptic plasticity, but also potentially provide an overview of neuronal network.

Mass spectrometry-based proteomics analysis

Mass spectrometry is the mainstream approach for identification and quantification of proteins and posttranslational modifications, with in small scale or in the entire proteome (Pagala, High et al. 2015). Individual peptide can be isolated from a mixture sample through multiple rounds of mass spectrometry, usually through molecule fragmentation. This gives out more accurate protein identification and a more comprehensive characterization of proteins with quantitative and qualitative changes. For example, by utilizing LC-MS/MS, a novel candidate synaptic ubiquitinated protein STIM1 was identified in the proteomic screening (Keil, Shen et al. 2010). Furthermore, proteomic techniques that are used for characterizing specific sites of ubiquitination in a large-scale fashion have been well established. Researchers found that all of the ubiquitinated proteins contain two C-terminal glycine residues of ubiquitin after digestion with trypsin; they took advantage of this di-glycine site and identify ubiquitination sites of digested substrate proteins by the utilization of di-glycine antibody, which specifically

recognize and immunoprecipiate peptides containing the modified lysine residues in digested substrate proteins. One large-scale proteomic study focuses on analysis of ubiquitinated proteome in rate brain uses similar monoclonal antibody that recognizes the di-glycine tag on lysine residues in trypsinized peptides (K-GG peptides). In this study, they reveals a wide range of ubiquitination events on key components in pre and post-synaptic region, and able to determine ubiquitination sites on key agents in several neurodegenerative diseases (Na and J.Peng 2012).

The main goal of this project is to look at the role of ubiquitin-dependent endocytosis and trafficking in the plasma membrane protein population, and systematically analyze the change of proteome in response to lysosomal inhibition in addition to identify key synaptic plasticity players that are regulated by the same mechanism as AMPAR. In the present study, we use biotinylation and quantitative mass spectrometry-based techniques (LC-MS/MS) to investigate the effects of a 12 h treatment of leupeptin and chloroquine on the proteome in cortical neurons. By perturbing ubiquitin-dependent protein degradation pathway such as the lysosomal pathway, we can identify membrane surface proteins that are regulated through ubiquitin-lysosomal degradation pathway. We hope to identify substrate-processing factors that act as critical mediators of the turnover of ubiquitin-dependent membrane proteins. Lastly, examining the change of plasma membrane proteins in different neuronal activity paradigms can provide us a better understanding of the role of ubiquitination in controlling surface membrane proteins, which is strongly related to neuronal development and function, and we aim to find novel targets of ubiquitin-dependent trafficking.

II. Results

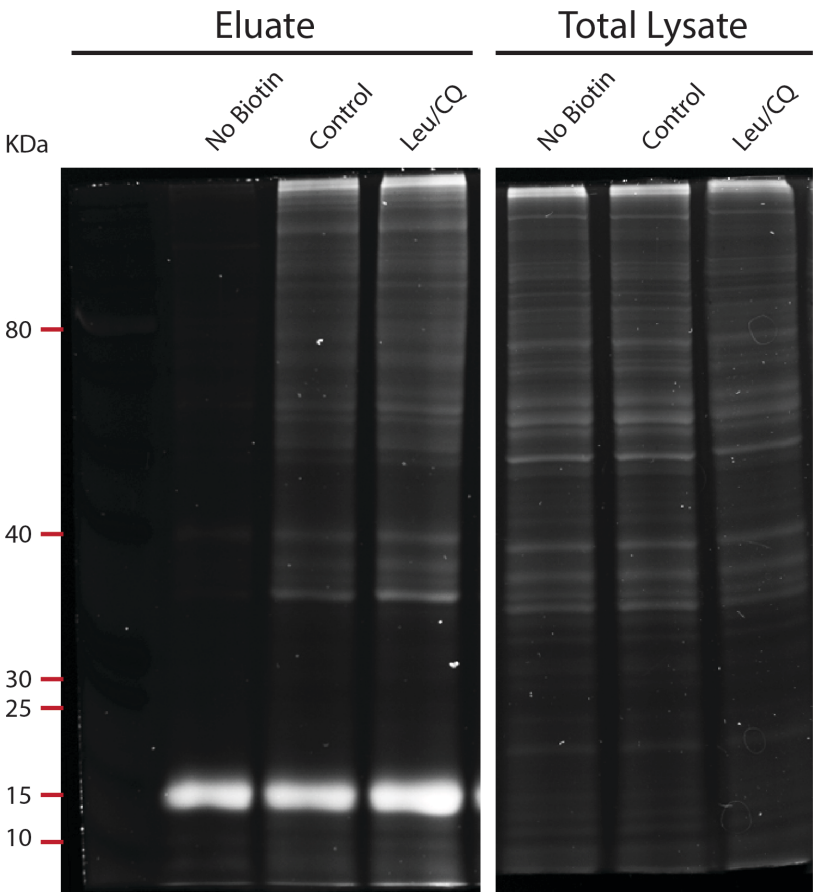


Figure 1: Total protein stains of synaptic fractions isolated from cortical neurons after 12 hr lysosomal inhibition with 200uM leupeptin and chloroquine. Cell membrane proteins of Synaptic fraction were separated by SDS-PAGE and visualized by staining fixed gels with Sypro Ruby.

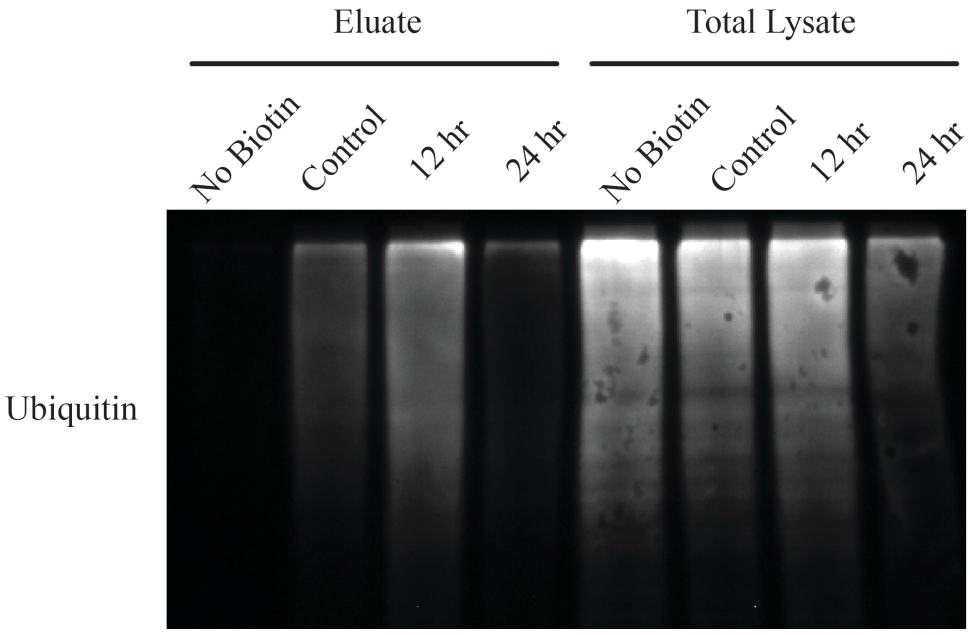


Figure 2: Ubiquitinated proteins on the cell membrane at the synapse after 12 and 24 hours lysosomal inhibition treatment with 200uM leupeptin and chloroquine. Biotin-avidin pulldown assays were performed on cortical neuron synaptosomal membranes. Eluted proteins were assayed for ubiquitin conjugates by anti-ubiquitin immunoblot.

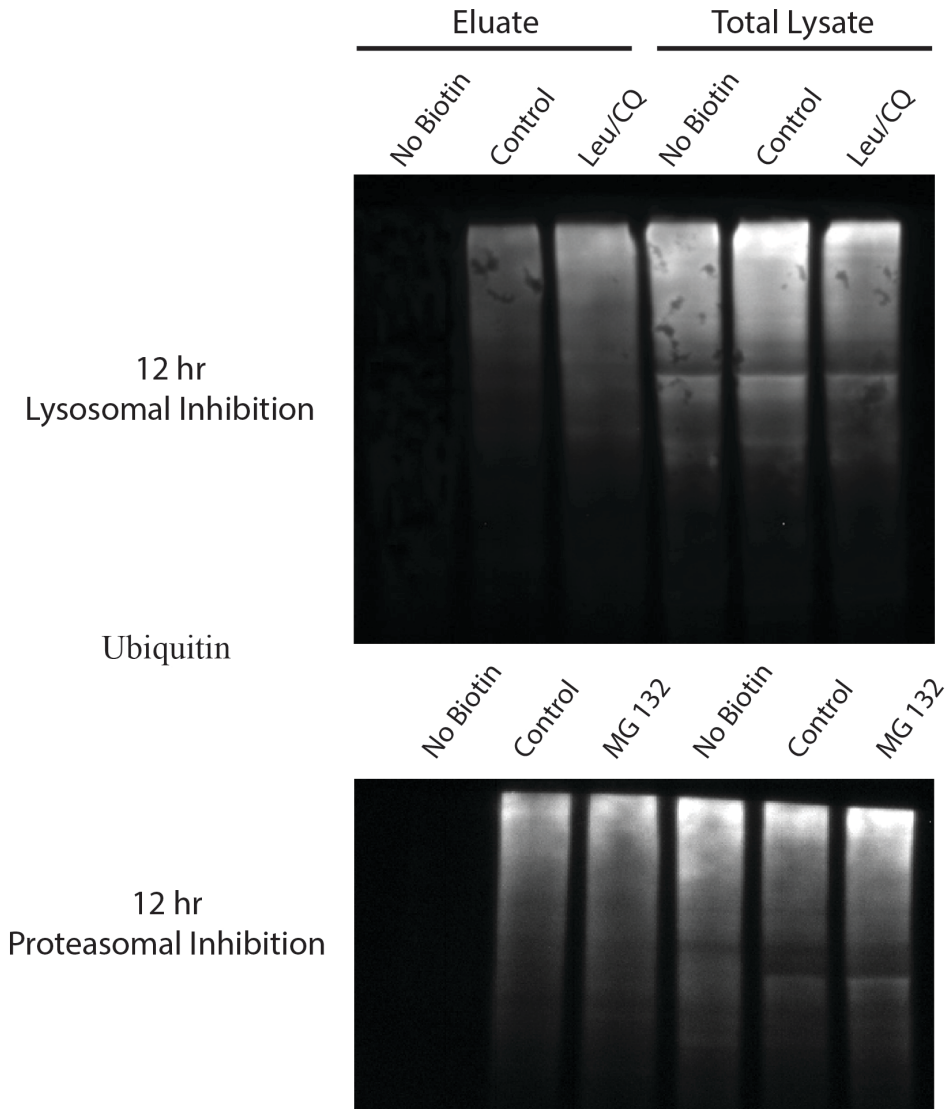


Figure 3: Ubiquitinated proteins on the cell membrane under lysosomal inhibition and proteasomal inhibition. Treated with 200uM leupeptin and 200uM chloroquine (top blot), and proteasomal inhibition treated with 25uM MG132 (bottom blot) for 12 hours.

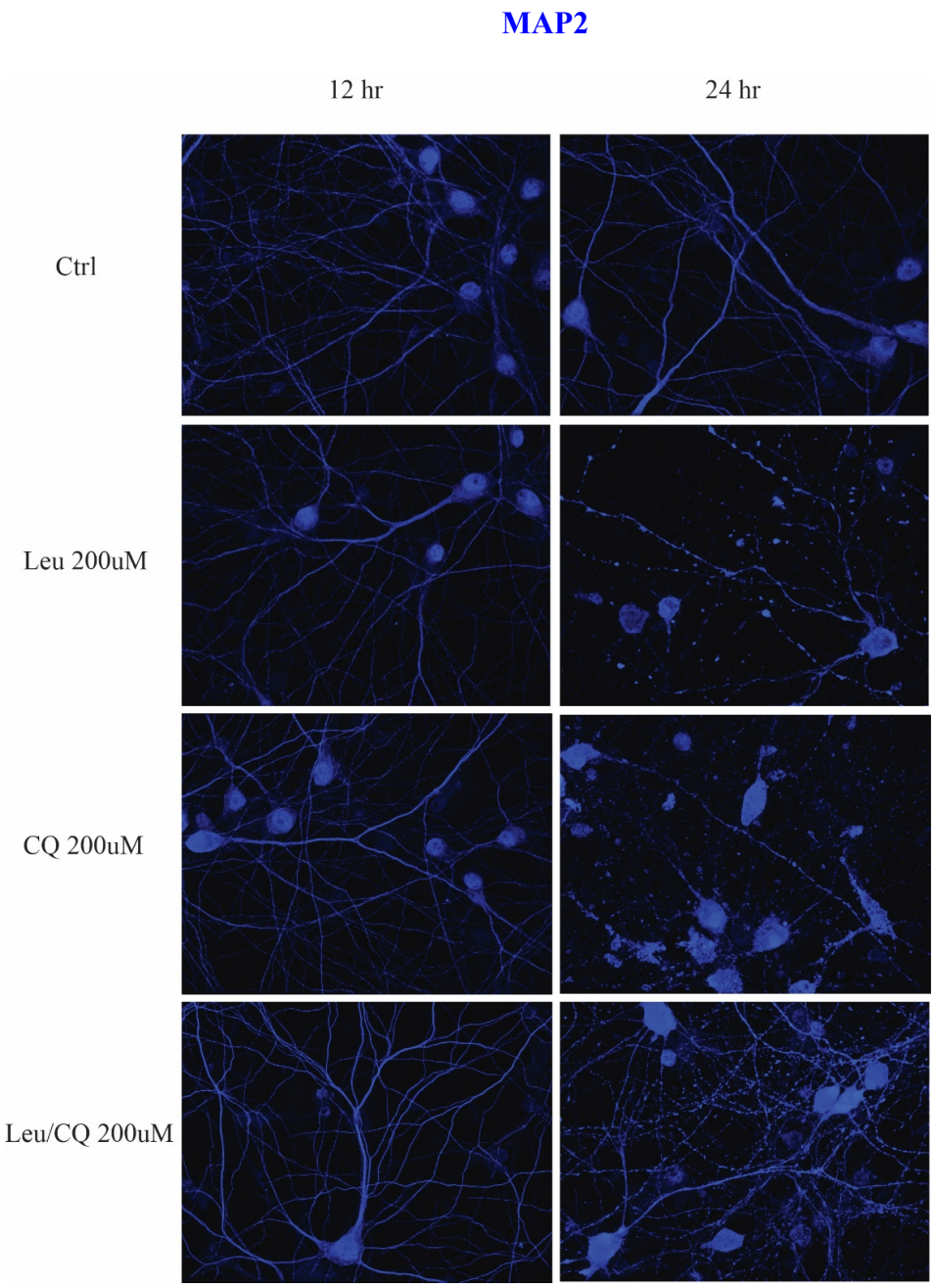


Figure 4: Cytotoxicity under lysosomal inhibition treatment for 12hr and 24hr**.** Immunocytochemical staining of neuronal dendrites marker, Microtubule-associated protein 2 (MAP2), shows the health of hippocampal neurons after 12hr and 24hr treated with 200uM leupeptin, 200uM chloroquine, and 200uM of leupeptin and chloroquine.

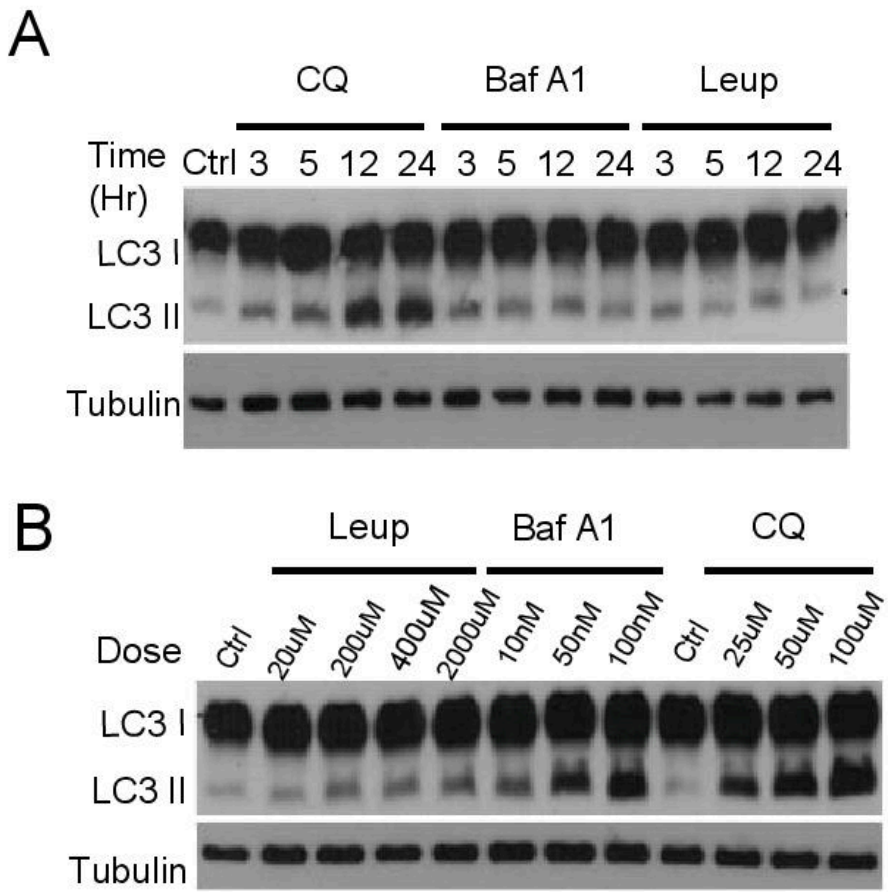


Figure 5: Induction of autophagy by lysosomal inhibitors time-dependent and dose-dependent in dissociated cortical neurons. (A) Neurons were treated with Chloroquine (25uM), Bafilomycin A1 (10nM), Leupeptin (200uM) for 3hrs, 5hrs, 12hrs, and 24hrs. (B) Neurons were treated with Leupeptin (20uM, 200uM, 400uM, 2000uM), Bafilomycin A1 (10nM, 50nM, 100nM), Chloroquine (25uM, 50uM, 100uM) for 12 hrs.

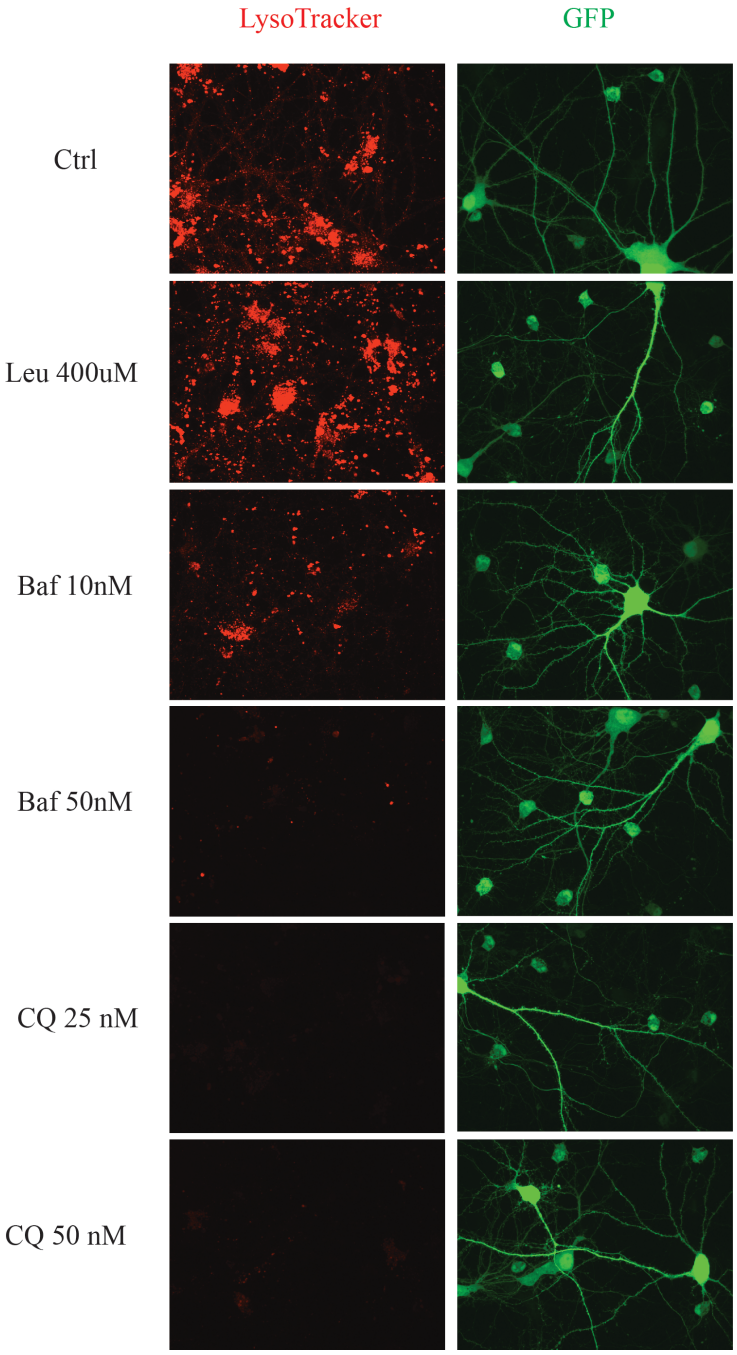
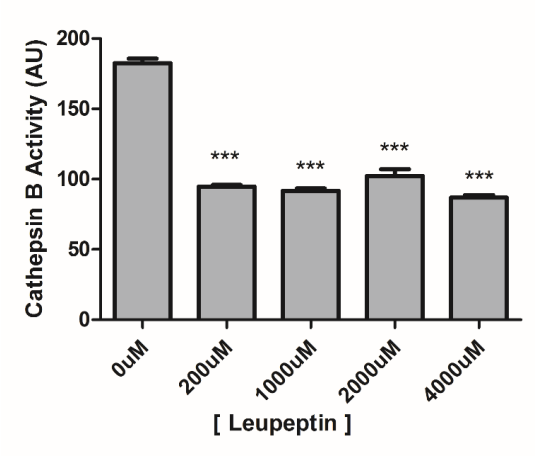


Figure 6: Acidification of lysosomes in the cells treated with leupeptin, bafilomycin, and chloroquine. (A) Hippocampal neurons were treated with leupeptin (400uM), bafilomycin A1 (10nM, 50nM), chloroquine (25uM, 50uM) for 12 hrs. Cells were then stained with LysoTracker Red DND-99 in MatTeks (70nM) for 30 min. Immunofluorescent images of dissociated hippocampal neurons expressing GFP and LysoTracker signals.



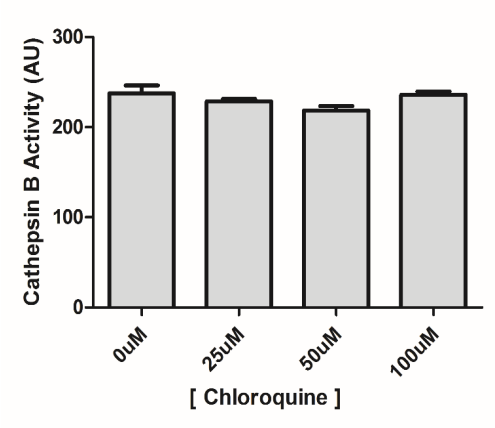


Figure 7: Lysosomal enzymatic activity under drug treatments. To determine the inhibition level of leupeptin and chloroquine on lysosomal enzyme Cathepsin B function, in vitro Cathepsin B activity was measured with the fluorogenic Cathepsin B Z-RR-AMC (fluorophore) was used. Increasing leupeptin concentration decreases enzymatic activity as measured by AMC hydrolysis. ($P<0.0001$).

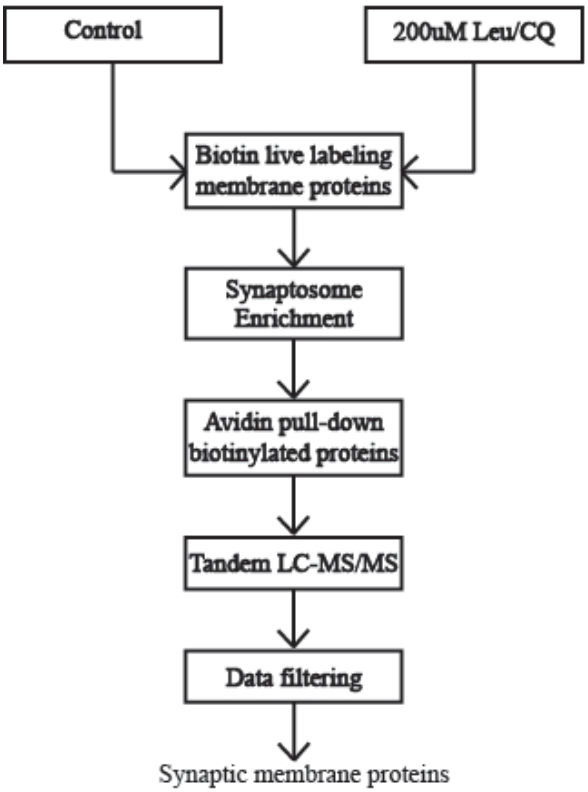
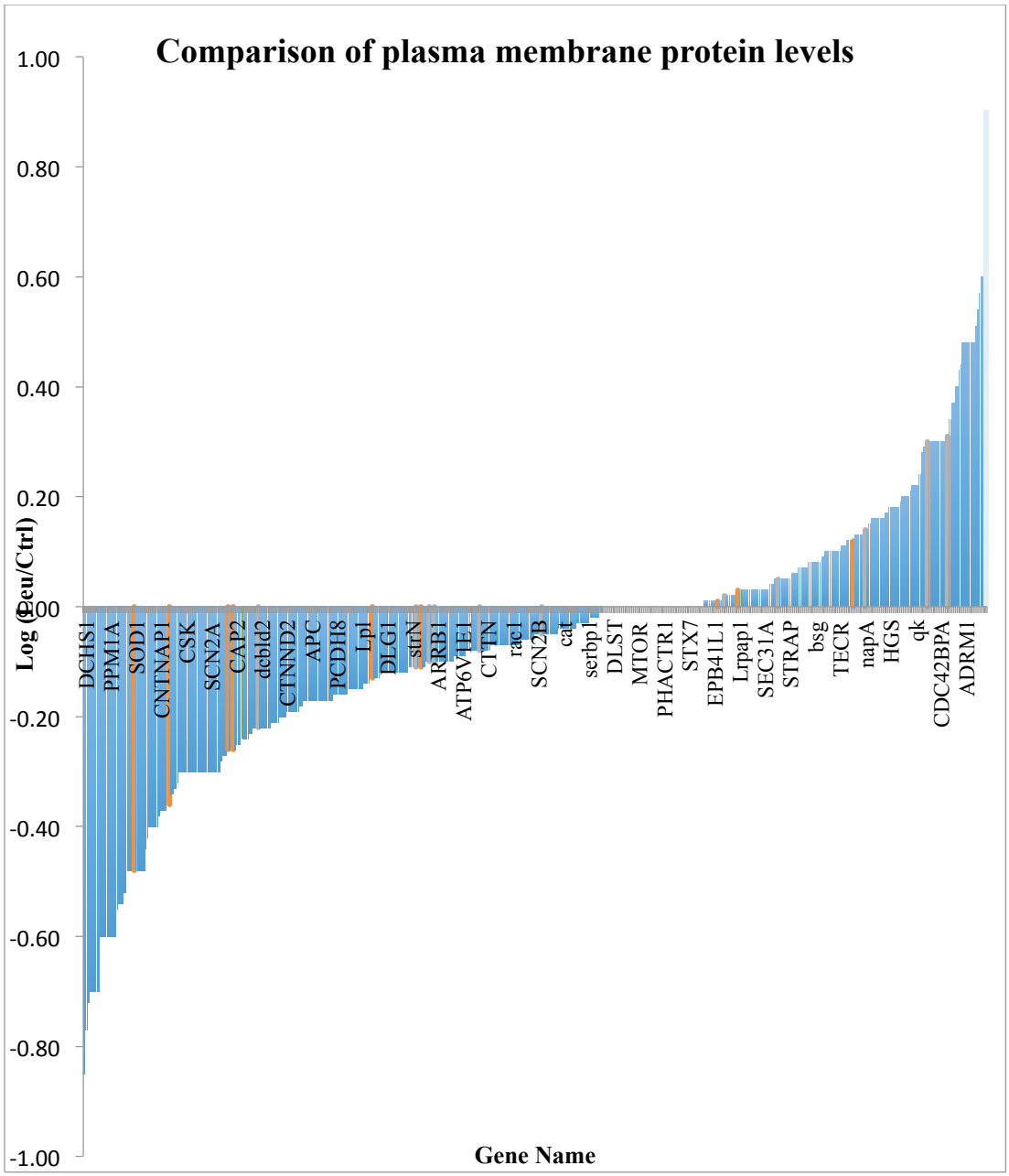


Figure 8: Schematized overview of proteomic screen for the isolation and identification of synaptic membrane proteins from hippocampal neurons treated with lysosomal inhibitors and control conditions.



Note:
Red bars: Proteins that are identified as synaptic candidate ubiquitinated proteins (Keil and Patrick 2010).
Green bars: Proteins that are identified as synaptic candidate ubiquitinated proteins and contain identified ubiquitination sites (Na and J.Peng 2012).

Figure 9: Bar graph shows plasma membrane protein levels between normal and lysosomal inhibited conditions by the spectral count ratio. Out of these plasma membrane proteins, we found that 105 of them were upregulated, and 207 were downregulated, while 226 did not change in protein level.

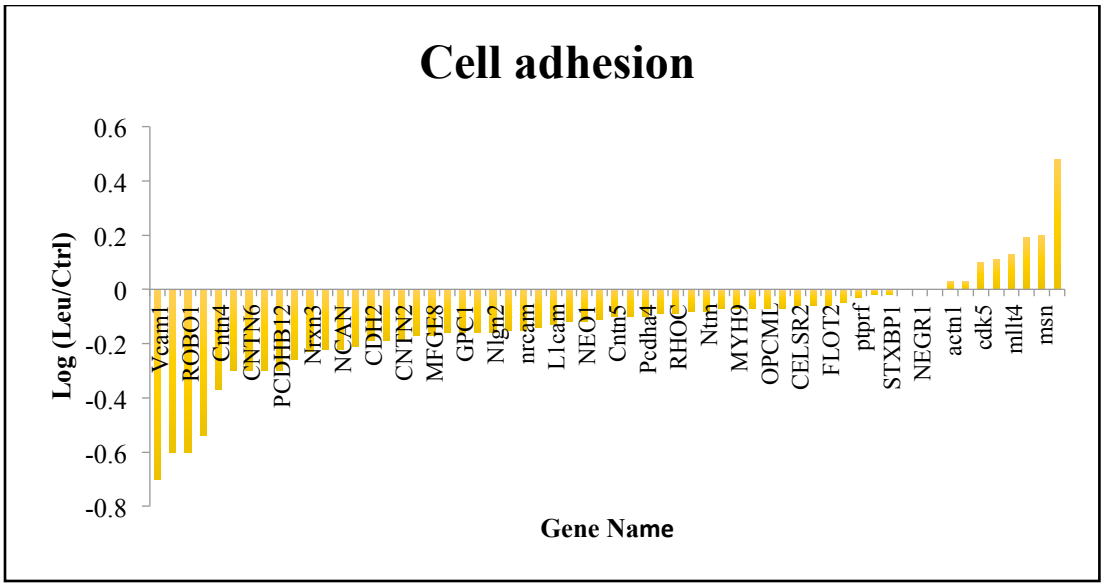


Figure 10: Bar graph shows cell adhesion protein levels at the synaptic membrane between normal and lysosomal inhibited conditions by the spectral count ratio.

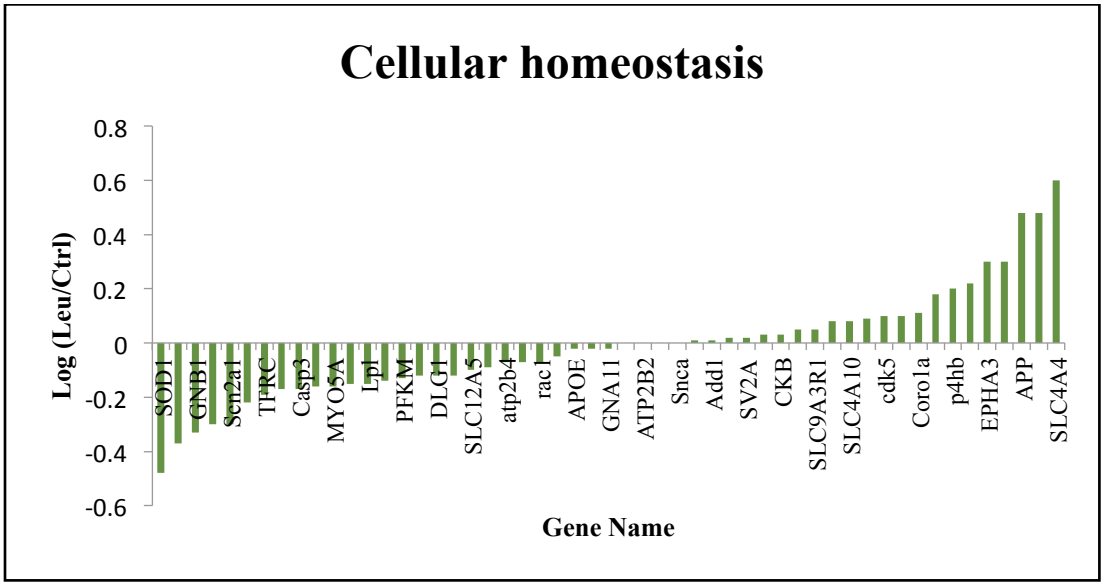


Figure 11: Bar graph shows cellular homeostasis regulators protein levels at the synaptic membrane between normal and lysosomal inhibited conditions by the spectral count ratio.

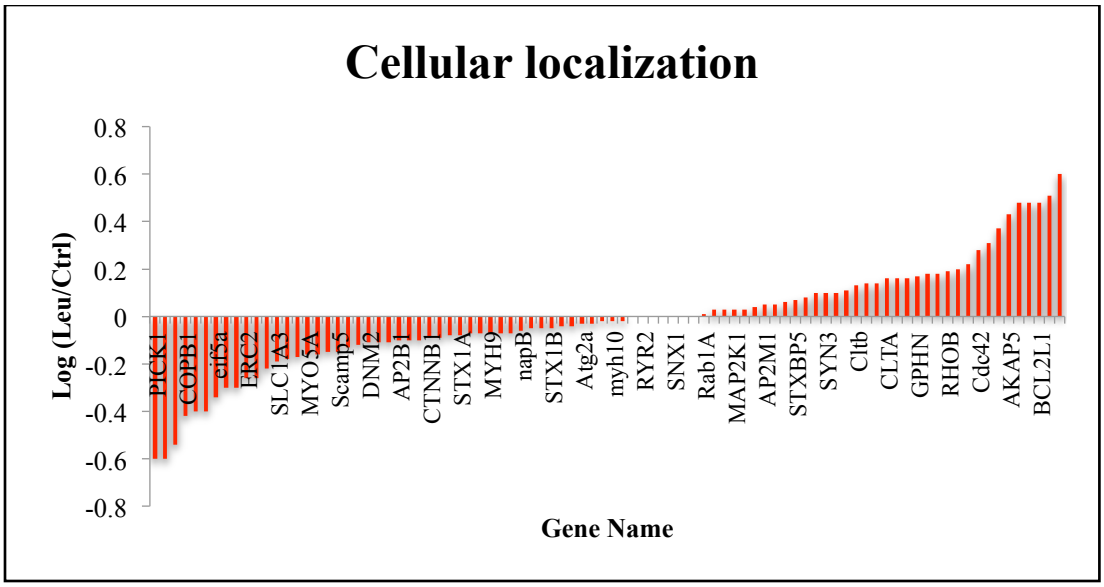


Figure 12: Bar graph shows cellular localization regulators protein levels at the synaptic membrane between normal and lysosomal inhibited conditions by the spectral count ratio.

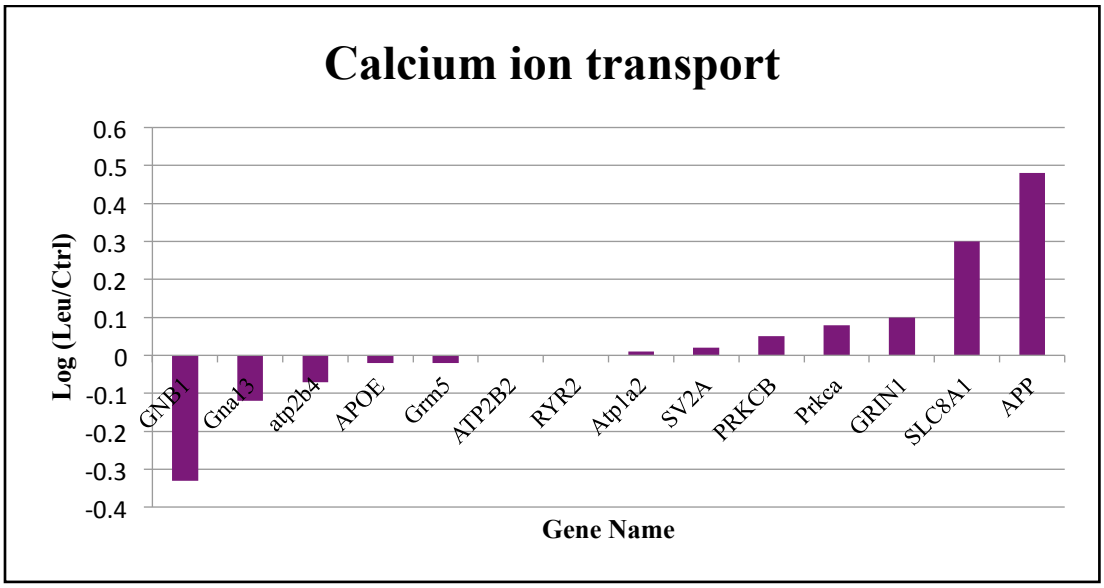


Figure 13: Bar graph Bar graph shows calcium ion transport regulators protein levels at the synaptic membrane between normal and lysosomal inhibited conditions by the spectral count ratio.

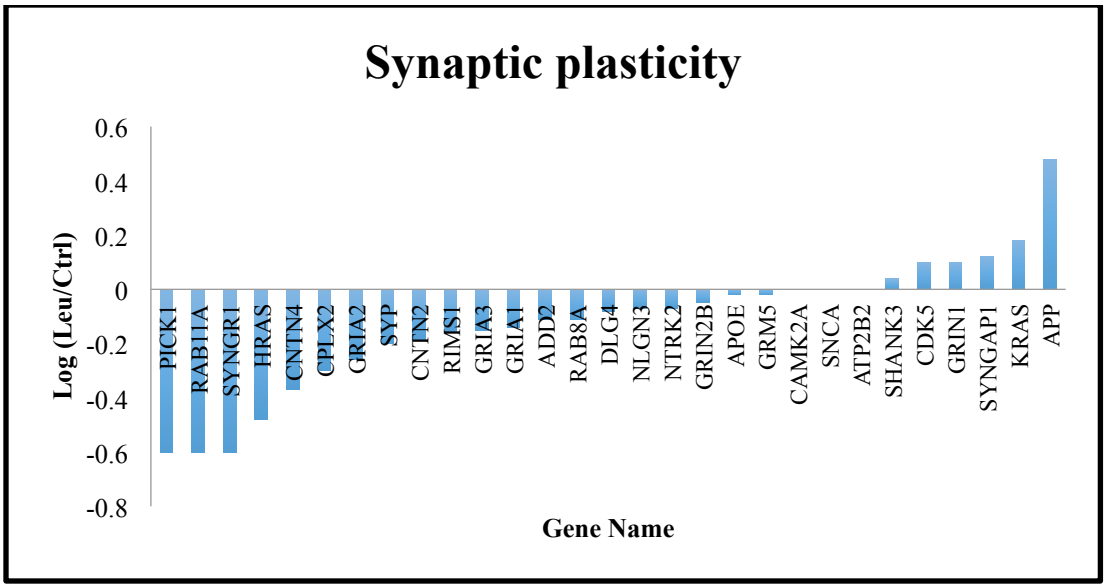


Figure 14: Bar graph Bar graph shows synaptic plasticity regulators protein levels at the synaptic membrane between normal and lysosomal inhibited conditions by the spectral count ratio.

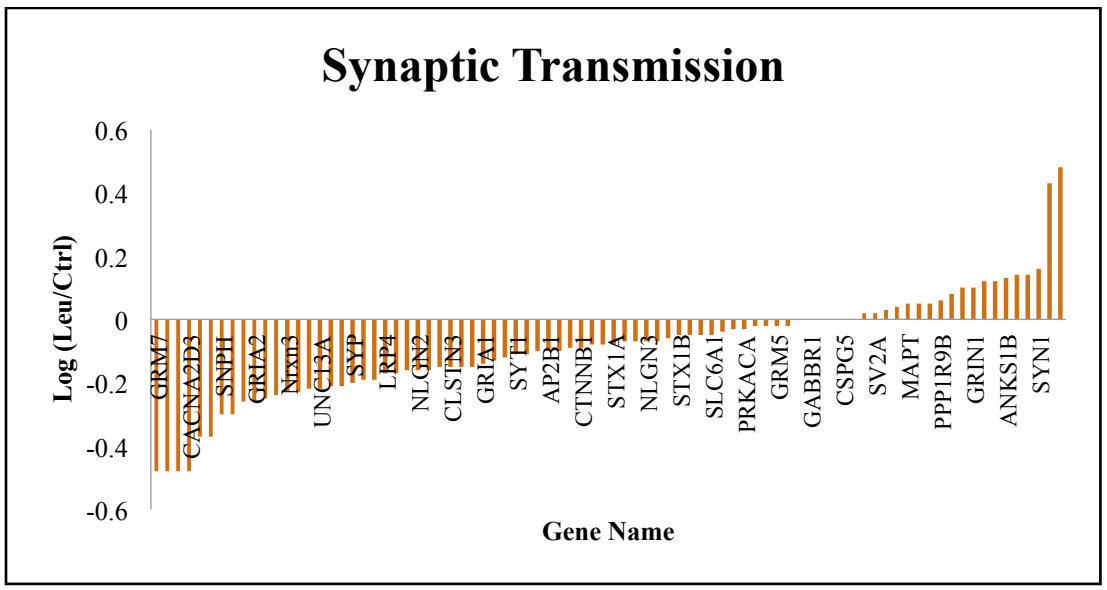


Figure 15: Bar graph Bar graph shows synaptic transmission regulators protein levels at the synaptic membrane between normal and lysosomal inhibited conditions by the spectral count ratio.

Table 1: Summary of functional categories of detected plasma membrane proteins by DAVID and IPA.

Type	Total (David)	Total (IPA)	Percentage
Enzyme	95	9	19%
G-protein coupled receptor	12	2	3%
Growth factor	1	0	0%
Ion channel	28	3	6%
Kinase	46	3	9%
Other	152	50	38%
Peptidase	6	1	1%
Phosphatase	6	7	2%
Transcription regulator	11	0	2%
Translation regulator	2	0	0%
Transmembrane receptor	7	5	2%
Transporter	82	11	17%
Grand Total	**448**	**91**	100%

Table 2: Plasma membrane proteins identified by DAVID and IPA					
UNIPROT Number	Official Gene Symbol	Gene Name	Ctrl SC	Leu SC	Ratio
Enzyme					
Q04400	adcy5	adenylate cyclase 5	5	3	0.60
P63245	GNB2L1	guanine nucleotide binding protein (G protein), beta polypeptide 2 like 1	7.5	1.5	0.20
Q9EP80	PICK1	protein interacting with PRKCA 1	4	1	0.25
Q62904	hsd17b7	hydroxysteroid (17-beta) dehydrogenase 7	4	1	0.25
P62494	Rab11a	RAB11a, member RAS oncogene family	4	1	0.25
P20171	hras	similar to GTPase HRas precursor (Transforming protein p21) (p21ras) (H-Ras-1) (c-H-ras); Harvey rat sarcoma virus oncogene	10.5	3.5	0.33
*P07632	SOD1	superoxide dismutase 1, soluble	3	1	0.33
O35353	GNB1	guanine nucleotide binding protein (G protein), beta polypeptide 1; guanine nucleotide binding protein (G protein), beta polypeptide 4	9.5	4.5	0.47
Q62845	Cntn4	contactin 4	28	12	0.43
Q6AYG3	PRUNE	prune homolog (Drosophila)	9	4	0.44
P61751	ARF4	ADP-ribosylation factor 4	13	6	0.46
P97528	CNTN6	contactin 6	6	3	0.50
156275	GGT7	gamma-glutamyltransferase 7	2	1	0.50
P49803	rgs7	regulator of G-protein signaling 7	7.5	4.5	0.60
P62836	RAP1A	RAP1A, member of RAS oncogene family	6.5	7	1.08
P26769	ADCY2	adenylate cyclase 2 (brain)	5	3	0.60
P10824	GNAI1	guanine nucleotide binding protein (G protein), alpha inhibiting 1; guanine nucleotide binding protein (G protein), alpha inhibiting 3	24.5	14.5	0.59
P09527	RAB7A	RAB7A, member RAS oncogene family	16.5	11.5	0.70
Q04970	NRAS	neuroblastoma ras oncogene	16	10	0.63
P62882	GNB5	guanine nucleotide binding protein (G protein), beta 5	4.5	3	0.67
P35281	RAB10	RAB10, member RAS oncogene family	13.5	8.5	0.63
P70478	APC	adenomatous polyposis coli	3	2	0.67
P23711	Hmox2	heme oxygenase (decycling) 2	6	4	0.67
Q63941	RAB3B	RAB3B, member RAS oncogene family	12	8	0.67
366227	APMAP	adipocyte plasma membrane associated	3	2	0.67

Table 2. (*Continued)*					
29254	MGLL	monoglyceride lipase	3	2	0.67
Q06000	Lpl	lipoprotein lipase	7	5	0.71
P84083	arf5	ADP-ribosylation factor 5	12	7	0.58
Q62888	Nlgn2	neuroligin 2	13	9	0.69
P19627	GNAZ	guanine nucleotide binding protein (G protein), alpha z polypeptide	19	14	0.74
Q9JJM9	GP1BB	glycoprotein Ib (platelet), beta polypeptide; septin 5	18	15.5	0.86
P35280	RAB8A	RAB8A, member RAS oncogene family	13	10	0.77
Q6Q7Y5	Gna13	guanine nucleotide binding protein, alpha 13	10.5	8	0.76
P39052	DNM2	dynamin 2	9	7	0.78
Q9QYF3	MYO5A	myosin Va	44	30.5	0.69
Q62889	NLGN3	neuroligin 3	29.5	25	0.85
P61227	RAP2B	RAP2B, member of RAS oncogene family	5	4	0.80
P70550	RAB8B	RAB8B, member RAS oncogene family	15	12	0.80
Q9WU34	septin 3	septin 3	11.5	7.5	0.65
*Q63198	Cntn1	contactin 1	223	175	0.78
P61589	RHOC	ras homolog gene family, member A; ras homolog gene family, member C	13.5	11	0.81
Q62952	Dpysl3	dihydropyrimidinase-like 3	90.5	75	0.83
**P59215	GNAO1	guanine nucleotide binding protein (G protein), alpha activating activity polypeptide O	113.5	94	0.83
294930	NCEH1	neutral cholesterol ester hydrolase 1	12	10	0.83
Q99ML5	PCYOX1	prenylcysteine oxidase 1	5.5	4.5	0.82
P62909	rps3	similar to 40S ribosomal protein S3; ribosomal protein S3	45	26	0.58
O35049	Smpd3	sphingomyelin phosphodiesterase 3, neutral	5.5	6	1.09
P04764	eno1	enolase 1, alpha pseudogene; similar to Alpha enolase (2-phospho-D-glycerate hydro-lyase); enolase 1, (alpha); similar to Alpha-enolase (2-phospho-D-glycerate hydro-lyase) (Non-neural enolase) (NNE) (Enolase 1)	67	60.5	0.90
Q9JID2	GNA11	guanine nucleotide binding protein, alpha 11	12	11.5	0.96
P61107	Rab14	RAB14, member RAS oncogene family	22	19	0.86
P04762	cat	catalase	27.5	25	0.91
Q62812	MYH9	myosin, heavy chain 9, non-muscle	49	41.5	0.85
Q6RUV5	rac1	ras-related C3 botulinum toxin substrate 1	21	18	0.86

Table 2. (*Continued)*					
Q05683	GAD2	glutamate decarboxylase 2	13	12	0.92
Q5U316	RAB35	RAB35, member RAS oncogene family	10.5	9.5	0.90
P63012	RAB3A	RAB3A, member RAS oncogene family	24.5	21	0.86
Q01205	DLST	dihydrolipoamide S-succinyltransferase (E2 component of 2-oxo-glutarate complex)	5.5	5.5	1.00
Q99PW3	Neu1	sialidase 1 (lysosomal sialidase)	8	8	1.00
Q63364	pja2	praja 2, RING-H2 motif containing	1.5	1.5	1.00
P22734	COMT	catechol-O-methyltransferase	2	2	1.00
P37377	Snca	synuclein, alpha (non A4 component of amyloid precursor)	36	36	1.00
P07323	eno2	enolase 2, gamma, neuronal	39	39	1.00
83512	FADS2	fatty acid desaturase 2	2	2	1.00
P82471	GNAQ	guanine nucleotide binding protein, alpha q polypeptide	28	29.5	1.05
Q6NYB7	Rab1A	similar to RAB1, member RAS oncogene family; RAB1, member RAS oncogene family	27.5	28	1.02
P63322	RALA	v-ral simian leukemia viral oncogene homolog A (ras related)	19	20	1.05
116743	SH3GL2	SH3-domain GRB2-like 2	21	21	1.00
P51635	AKR1A1	aldo-keto reductase family 1, member A1 (aldehyde reductase)	17.5	16.5	0.94
Q6GQP4	rab31	RAB31, member RAS oncogene family	4	3.5	0.88
P62824	Rab3c	RAB3C, member RAS oncogene family	16	14.5	0.91
Q62940	NEDD4	neural precursor cell expressed, developmentally down-regulated gene 4	4	4.5	1.13
P63095	Gnas	GNAS complex locus	22	22.5	1.02
P00507	Got2	similar to Aspartate aminotransferase, mitochondrial precursor (Transaminase A) (Glutamate oxaloacetate transaminase 2); glutamic-oxaloacetic transaminase 2, mitochondrial (aspartate aminotransferase 2)	66	57	0.86
P54313	gnb2	guanine nucleotide binding protein (G protein), beta polypeptide 2	19	17.5	0.92
O35509	RAB11B	RAB11B, member RAS oncogene family	6	7	1.17
P05714	rab4a	RAB4A, member RAS oncogene family	5	6	1.20
499913	ABHD12	abhydrolase domain containing 12	6	7	1.17
P20070	cyb5r3	cytochrome b5 reductase 3	11	11	1.00
Q9Z1A5	Nae1	NEDD8 activating enzyme E1 subunit 1	4	5	1.25
O35274	Ppp1r9b	protein phosphatase 1, regulatory subunit 9B	33.5	39	1.16

Table 2. (*Continued)*					
Q566R0	them4	thioesterase superfamily member 4	3	4	1.33
P35286	RAB13	RAB13, member RAS oncogene family	5	7	1.40
Q91Y81	Septin-2	similar to Septin-2 (Protein NEDD5) (Neural precursor cell expressed developmentally down-regulated protein 5); septin 2	6	9.5	1.58
Q03555	GPHN	gephyrin	13.5	20	1.48
Q99P75	RAB9A	RAB9A, member RAS oncogene family	2	3	1.50
P08644	Kras	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog	6	9	1.50
P18163	ACSL1	acyl-CoA synthetase long-chain family member 1	5	8	1.60
P51146	RAB4B	RAB4B, member RAS oncogene family	8	13	1.63
191576	TECR	trans-2,3-enoyl-CoA reductase	4	5	1.25
P04785	p4hb	prolyl 4-hydroxylase, beta polypeptide	30.5	48	1.57
P63039	HSPD1	similar to 60 kDa heat shock protein, mitochondrial precursor (Hsp60) (60 kDa chaperonin) (CPN60) (Heat shock protein 60) (HSP-60) (Mitochondrial matrix protein P1) (HSP-65); similar to 60 kDa heat shock protein, mitochondrial precursor (Hsp60); heat shock protein 1 (chaperonin); similar to heat shock protein 1 (chaperonin)	42	115	2.74
Q8CFN2	Cdc42	cell division cycle 42 (GTP binding protein)	11.5	22	1.91
500282	ARL8B	ADP ribosylation factor like GTPase 8B	7	6	0.86
Q6P7C7	GPNMB	glycoprotein (transmembrane) nmb	2	1	0.50
Q6MGB5	HSD17B8	hydroxysteroid (17-beta) dehydrogenase 8	1	2	2.00
P97538	mras	muscle RAS oncogene homolog	4	8	2.00
P21396	MAOA	monoamine oxidase A	13	14	1.08
P37996	Arl3	ADP-ribosylation factor-like 3	3	1	0.33
Q62639	rheb	Ras homolog enriched in brain	1	3	3.00
P62747	RHOB	ras homolog gene family, member B	9	14	1.56
Q91ZY8	TRIM9	tripartite motif-containing 9	1	4	4.00
P24329	TST	thiosulfate sulfurtransferase, mitochondrial	1	8	8.00
P30839	aldh3a2	aldehyde dehydrogenase 3 family, member A2	5	2	0.40
O35547	Acsl4	acyl-CoA synthetase long-chain family member 4	21	21	1.00
G-protein coupled receptor					
P35400	GRM7	glutamate receptor, metabotropic 7	6	2	0.33

Table 2. (*Continued)*					
P35365	Htr5b	5-hydroxytryptamine (serotonin) receptor 5B	2	1	0.50
O88917	ADGRL1	latrophilin 1	36	18	0.50
291352	GPR158	G protein-coupled receptor 158	25	17	0.68
O88278	Celsr3	cadherin, EGF LAG seven-pass G-type receptor 3 (flamingo homolog, Drosophila)	13	9	0.69
Q9Z173	ADGRL3	latrophilin 3	36	24	0.67
P22909	Adra2a	adrenergic, alpha-2A-, receptor	4	3	0.75
Q8K3V3	GPR56	G protein-coupled receptor 56	6.5	6.5	1.00
Q9QYP2	CELSR2	cadherin, EGF LAG seven-pass G-type receptor 2 (flamingo homolog, Drosophila)	15	13	0.87
171447	ADGRL2	adhesion G protein-coupled receptor L2	10	9	0.90
O88923	ADGRL2	latrophilin 2	10	9	0.90
O88871	Gabbr2	gamma-aminobutyric acid (GABA) B receptor 2	13.5	10	0.74
P31424	Grm5	glutamate receptor, metabotropic 5	9.5	9	0.95
Q9Z0U4	GABBR1	gamma-aminobutyric acid (GABA) B receptor 1	5.5	5.5	1.00
Q9ERQ6	cspg5	chondroitin sulfate proteoglycan 5	3	3	1.00
Ion channel					
Q8CFG5	CACNA2D3	calcium channel, voltage-dependent, alpha2/delta subunit 3	6	2	0.33
Q62976	KCNMA1	potassium large conductance calcium-activated channel, subfamily M, alpha member 1	7	3	0.43
P20236	GABRA3	gamma-aminobutyric acid (GABA) A receptor, alpha 3	7	3	0.43
306439	GPM6A	glycoprotein M6A	6	3	0.50
Q8CFG6	CACNA2D2	calcium channel, voltage-dependent, alpha 2/delta subunit 2	8	4	0.50
P04775	Scn2a1	sodium channel, voltage-gated, type II, alpha 1	10	5	0.50
24766	SCN2A	sodium channel, voltage gated, type II alpha subunit	10	5	0.50
P19491	GRIA2	glutamate receptor, ionotropic, AMPA 2	38	21	0.55
P62813	Gabra1	gamma-aminobutyric acid (GABA) A receptor, alpha 1	13	8	0.62
P0C5X8	ttyh1	tweety homolog 1 (Drosophila)	7.5	5.5	0.73
P19492	Gria3	glutamate receptor, ionotrophic, AMPA 3	10	7	0.70
P19490	GRIA1	glutamate receptor, ionotropic,	25	18	0.72

Table 2. (*Continued)*					
*P54290	Cacna2d1	calcium channel, voltage-dependent, alpha2/delta subunit 1	70	51.5	0.74
P19493	GRIA4	glutamate receptor, ionotropic, AMPA4	5	4	0.80
P54900	SCN2B	sodium channel, voltage-gated, type II, beta	4.5	4	0.89
**Q9Z2L0	VDAC1	voltage-dependent anion channel 1; similar to Voltage-dependent anion-selective channel protein 1 (VDAC-1) (Outer mitochondrial membrane protein porin); similar to Voltage-dependent anion-selective channel protein 1 (VDAC-1) (mVDAC1) (mVDAC5) (Outer mitochondrial membrane protein porin 1) (Plasmalemmal porin)	30.5	23.5	0.77
Q8VHW5	Cacng8	calcium channel, voltage-dependent, gamma subunit 8	7.5	7	0.93
P48037	ANXA6	annexin A6	12.5	12.5	1.00
Q00960	GRIN2B	glutamate receptor, ionotropic, N-methyl D-aspartate 2B	13.5	12	0.89
Q9Z0W7	CLIC4	chloride intracellular channel 4 (mitochondrial)	1.5	1.5	1.00
B0LPN4	RYR2	ryanodine receptor 2, cardiac	5	5	1.00
Q7TP36	SHROOM 2	shroom family member 2	5	5	1.00
Q91XV6	fxyd6	FXYD domain-containing ion transport regulator 6	5	5	1.00
29738	KCNAB2	potassium channel, voltage gated subfamily A regulatory beta subunit 2	5	5	1.00
P54287	CACNB3	calcium channel, voltage-dependent, beta 3 subunit	9	7.5	0.83
P54283	CACNB1	calcium channel, voltage-dependent, beta 1 subunit	9	9.5	1.06
P35439	GRIN1	glutamate receptor, ionotropic, N-methyl D-aspartate 1	8	10	1.25
P63079	GABRB3	gamma-aminobutyric acid (GABA) A receptor, beta 3	9	13	1.44
Q9WUD2	TRPV2	transient receptor potential cation channel, subfamily V, member 2	4	6	1.50
P15387	KCNB1	potassium voltage gated channel, Shab-related subfamily, member 1	4	7	1.75
P84903	STIM1	stromal interaction molecule 1	1	3	3.00
Kinase					
Q03351	NTRK3	neurotrophic tyrosine kinase,	3	2	0.67

Table 2. (*Continued)*					
O35276	NRP2	neuropilin 2	17	5	0.29
P32577	CSK	c-src tyrosine kinase	4	2	0.50
O55173	PDPK1	3-phosphoinositide dependent protein kinase-1	4	2	0.50
Q62956	Erbb4	v-erb-a erythroblastic leukemia viral oncogene homolog 4 (avian)	5	3	0.60
29202	EPHA6	EPH receptor A6	5	3	0.60
P97523	MET	met proto-oncogene	3	2	0.67
P19804	NME2	non-metastatic cells 2, protein (NM23B) expressed in; non-metastatic cells 2, protein (NM23B) expressed in, pseudogene 1	24.5	17	0.69
Q62696	DLG1	discs, large homolog 1 (Drosophila)	8	6	0.75
**Q62915	CASK	calcium/calmodulin-dependent serine protein kinase (MAGUK family)	16	12	0.75
P47858	PFKM	phosphofructokinase, muscle	23	17	0.74
Q63092	CAMKV	CaM kinase-like vesicle-associated	22.5	17	0.76
P12368	PRKAR2A	protein kinase, cAMP dependent regulatory, type II alpha	20.5	18	0.88
P31016	DLG4	discs, large homolog 4 (Drosophila)	20.5	17	0.83
Q63622	Dlg2	discs, large homolog 2 (Drosophila)	17	14.5	0.85
Q63604	NTRK2	neurotrophic tyrosine kinase, receptor, type 2	7	6	0.86
B2DD29	Brsk1	BR serine/threonine kinase 1	6.5	4	0.62
Q9Z272	GIT1	G protein-coupled receptor kinase interacting ArfGAP 1	17	15.5	0.91
P07335	CKB	creatine kinase, brain	36	38.5	1.07
P27791	PRKACA	similar to CG2662-PA; protein kinase, cAMP-dependent, catalytic, alpha	18	17	0.94
Q62868	ROCK2	Rho-associated coiled-coil containing protein kinase 2	4	3	0.75
**P15791	CAMK2D	calcium/calmodulin-dependent protein kinase II delta	43.5	46	1.06
P42346	MTOR	mechanistic target of rapamycin (serine/threonine kinase)	3	3	1.00
Q62833	GRK5	G protein-coupled receptor kinase 5	4	4	1.00
79208	EPHA5	EPH receptor A5	7	7	1.00
P09216	Prkce	protein kinase C, epsilon	9	9.5	1.06
**P11275	Camk2a	calcium/calmodulin-dependent protein kinase II alpha	67.5	68.5	1.01
P39069	ak1	adenylate kinase 1	35	36	1.03
Q01986	MAP2K1	mitogen activated protein kinase	12	13	1.08

Table 2. (*Continued)*					
P68403	PRKCB	protein kinase C, beta	12.5	14	1.12
P0C1X8	aak1	AP2 associated kinase 1	6.5	7.5	1.15
Q62936	DLG3	discs, large homolog 3 (Drosophila)	40	43	1.08
Q62844	FYN	FYN oncogene related to SRC, FGR, YES	11.5	10.5	0.91
P26817	ADRBK1	adrenergic, beta, receptor kinase 1	7	8	1.14
O88382	MAGI2	membrane associated guanylate kinase, WW and PDZ domain containing 2	5	6	1.20
Q03114	cdk5	cyclin-dependent kinase 5	8	10	1.25
P05696	Prkca	protein kinase C, alpha	14.5	17.5	1.21
Q4L1J4	MAGI1	membrane associated guanylate kinase, WW and PDZ domain containing 1	5.5	6.5	1.18
P05708	HK1	hexokinase 1	87	74	0.85
Q7TT49	CDC42BPB	CDC42 binding protein kinase beta (DMPK-like)	10.5	13.5	1.29
117542	BAIAP2	BAI1-associated protein 2	25	25.5	1.02
Q810W7	MAST1	microtubule associated serine/threonine kinase 1	2	3	1.50
O88377	PIP4K2B	phosphatidylinositol-5-phosphate 4-kinase, type II, beta	7.5	7.5	1.00
O08680	EPHA3	Eph receptor A3	1	2	2.00
O54874	CDC42BPA	CDC42 binding protein kinase alpha	2.5	5	2.00
Q9JK71	MAGI3	membrane associated guanylate kinase, WW and PDZ domain containing 3	3	6	2.00
P97874	GAK	cyclin G associated kinase	2	5	2.50
O88954	MPP3	membrane protein, palmitoylated 3 (MAGUK p55 subfamily member 3)	1	3	3.00
O08679	MARK2	MAP/microtubule affinity-regulating kinase 2	4	15	3.75
Other					
308912	DCHS1	dachsous cadherin-related 1	7	1	0.14
P51653	Gpc2	glypican 2	6	1	0.17
688721	LRFN4	leucine rich repeat and fibronectin type III domain containing 4	6	1	0.17
Q00657	cspg4	chondroitin sulfate proteoglycan 4	13	2.5	0.19
29602	PTGFRN	prostaglandin F2 receptor inhibitor	5	1	0.20
P0C7J6	LRFN1	leucine rich repeat and fibronectin type III domain containing 1	5	1	0.20
P60756	MDGA2	MAM domain containing glycosylphosphatidylinositol anchor	5	1	0.20

Table 2. (*Continued)*					
P30427	PLEC	plectin 1	4	1	0.25
365216	CADM4	cell adhesion molecule 4	4	1	0.25
100359982	MPC2	mitochondrial pyruvate carrier 2	4	1	0.25
24588	Nefm	neurofilament, medium polypeptide	9	2.5	0.28
P85171	MDGA1	MAM domain containing glycosylphosphatidylinositol anchor 1	7	2	0.29
O88339	epn1	Epsin 1	10	3	0.30
P52796	efnb1	ephrin B1	10	3	0.30
316205	LRFN2	leucine rich repeat and fibronectin type III domain containing 2	3	1	0.33
681989	VIPAS39	VPS33B interacting protein, apical-basolateral polarity regulator, spe-39 homolog	3	1	0.33
191571	FAT3	FAT atypical cadherin 3	22	8	0.36
84008	CNTNAP 1	contactin associated protein 1	6	2.5	0.42
Q62640	GRID1	glutamate receptor, ionotropic, delta 1	5	2	0.40
Q4V7C7	ACTR3	ARP3 actin-related protein 3 homolog (yeast)	18	14	0.78
Q1WIM2	CADM2	cell adhesion molecule 2	15	7	0.47
252892	LGI1	leucine-rich, glioma inactivated 1	10.5	5.5	0.52
Q4FZX7	SRPRB	signal recognition particle receptor, B subunit; transferrin	4	2	0.50
B5DF41	SNPH	syntaphilin	4	2	0.50
Q5FVC2	Arhgef2	rho/rac guanine nucleotide exchange factor (GEF) 2	4	2	0.50
P84087	CPLX2	complexin 2	16	8	0.50
Q9QYV1	TMEFF1	transmembrane protein with EGF-like and two follistatin-like domains 1	2	1	0.50
Q63418	PCDHB12	protocadherin beta 12	4	2	0.50
Q6P5P3	TTC9C	tetratricopeptide repeat domain 9C	1	2	2
366205	FLRT3	fibronectin leucine rich transmembrane protein 3	10	5	0.50
**Q9Z214	HOMER1	homer homolog 1 (Drosophila)	11.5	6.5	0.57
Q62682	CNTN3	contactin 3 (plasmacytoma associated)	9.5	6.5	0.68
P55280	CDH6	cadherin 6	7	4.5	0.64
P10536	RAB1B	RAB1B, member RAS oncogene family, pseudogene 1; RAB1B, member RAS oncogene family	13.5	7.5	0.56
Q8R491	ehd3	similar to EH-domain containing 3;	24	13	0.54

Table 2. (*Continued)*					
*Q8K3M6	ERC2	ELKS/RAB6-interacting/CAST family member 2	11	6	0.55
Q07310	Nrxn3	neurexin 3	43	25.5	0.59
P97527	Cntn5	contactin 5	25.5	20.5	0.80
P22063	CNTN2	contactin 2 (axonal)	20	13	0.65
O35112	Alcam	activated leukocyte cell adhesion molecule	5.5	3	0.55
Q6JP77	AKAP7	A kinase (PRKA) anchor protein 7	5	3	0.60
Q62768	UNC13A	unc-13 homolog A (C. elegans)	5	3	0.60
**P97836	dlgap1	discs, large (Drosophila) homolog-associated protein 1	5	3	0.60
Q91ZV2	dcbld2	discoidin, CUB and LCCL domain containing 2	5	3	0.60
314961	ASAP1	ArfGAP with SH3 domain, ankyrin repeat and PH domain 1	5	3	0.60
P04897	gnai2	guanine nucleotide binding protein (G protein), alpha inhibiting 2	26.5	16.5	0.62
P55067	NCAN	neurocan	10	6	0.60
P63170	dynll1	dynein light chain LC8-type 1	10	6.5	0.65
Q9Z1E1	flot1	flotillin 1	14.5	9.5	0.66
Q63537	Syn2	synapsin II	18.5	15	0.81
Q9Z1Y3	CDH2	cadherin 2	10	6.5	0.65
Q5BKC9	Ngef	neuronal guanine nucleotide exchange factor	3	2	0.67
25592	AGRN	agrin	3	2	0.67
Q9QYP1	LRP4	low density lipoprotein receptor-related protein 4	22	15	0.68
64865	PCDH8	protocadherin 8	29	20	0.69
25595	MAP2	microtubule associated protein 2	127	171.5	1.35
Q9JKE3	Scamp5	secretory carrier membrane protein 5	10	7	0.70
P70490	MFGE8	milk fat globule-EGF factor 8 protein	10.5	7	0.67
Q8R553	Clstn3	calsyntenin 3	7	5	0.71
298203	STOML2	stomatin like 2	7	4	0.57
O55012	PICALM	phosphatidylinositol binding clathrin assembly protein	9.5	7.5	0.79
P97685	Nfasc	neurofascin	70	48.5	0.69
Q5U2Y3	MPP7	membrane protein, palmitoylated 7 (MAGUK p55 subfamily member 7)	4	3	0.75
P97686	nrcam	neuronal cell adhesion molecule	81	57.5	0.71
64457	NDRG4	NDRG family member 4	12	9	0.75
117242	TENM2	teneurin transmembrane protein 2	21	16	0.76
Q05695	L1cam	L1 cell adhesion molecule	95	70.5	0.74

Table 2. (*Continued)*					
290364	ITM2B	integral membrane protein 2B	5	4	0.80
P29066	ARRB1	arrestin, beta 1	5	4	0.80
Q767I8	Pcdha4	protocadherin alpha 4	5	4	0.80
P70483	strN	striatin, calmodulin binding protein	6.5	5	0.77
360921	RUFY3	RUN and FYVE domain containing 3	23.5	16.5	0.70
288480	AIMP2	aminoacyl tRNA synthetase complex-interacting multifunctional protein 2	2.5	1	0.40
Q62625	MAP1LC 3B	microtubule-associated protein 1 light chain 3 beta	11	9	0.82
**P13596	Ncam1	neural cell adhesion molecule 1	194	154	0.79
O55043	Arhgef7	Rho guanine nucleotide exchange factor (GEF7)	10.5	9	0.86
25567	TNR	tenascin R	54	45.5	0.84
Q6P0K8	JUP	junction plakoglobin	6	5	0.83
P69682	Necap1	NECAP endocytosis associated 1	18	15	0.83
Q9JJ50	HGS	hepatocyte growth factor-regulated tyrosine kinase substrate	2	3	1.50
O08838	ampH	amphiphysin; similar to amphiphysin 1; similar to Amphiphysin	39	33	0.85
Q9Z1T4	CNKSR2	connector enhancer of kinase suppressor of Ras 2	6.5	7	1.08
60465	CTTN	cortactin	36.5	30.5	0.84
P32736	OPCML	opioid binding protein/cell adhesion molecule-like	21.5	18.5	0.86
Q78P75	Dynll2	dynein light chain LC8-type 2	16	14	0.88
Q9Z2S9	FLOT2	flotillin 2	16	14	0.88
P13852	PRNP	prion protein	10.5	8	0.76
P63025	vamp3	vesicle-associated membrane protein 3	8	8.5	1.06
Q9JJ19	SLC9A3R 1	solute carrier family 9 (sodium/hydrogen exchanger), member 3 regulator 1	15	17	1.13
Q1WIM3	CADM3	cell adhesion molecule 3	23	20.5	0.89
P11442	CLTC	clathrin, heavy chain (Hc)	178	161.5	0.91
**P07936	gap43	growth associated protein 43	53.5	47.5	0.89
Q62718	Ntm	neurotrimin	16	13.5	0.84
Q9Z270	vapa	VAMP (vesicle-associated membrane protein)-associated protein A	42.5	38.5	0.91
Q9Z0J8	NEGR1	neuronal growth regulator 1	20	20	1.00
Q4KM74	SEC22B	SEC22 vesicle trafficking protein homolog B (S. cerevisiae)	6	5.5	0.92

Table 2. (*Continued)*					
24851	Tpm1	tropomyosin 1, alpha	41	38	0.93
Q3B7U9	FKBP8	FK506 binding protein 8, 38kDa	4	3.5	0.88
29272	DPP6	dipeptidyl-peptidase 6	18.5	13	0.70
Q9JLT0	myh10	myosin, heavy chain 10, non-muscle	206.5	197	0.95
Q3KRD5	TOMM34	translocase of outer mitochondrial membrane 34	7.5	8	1.07
P04218	Cd200	Cd200 molecule	5.5	5	0.91
Q6AXS5	serbp1	Serpine1 mRNA binding protein 1	8	7.5	0.94
Q5XI73	ARHGDIA	Rho GDP dissociation inhibitor (GDI) alpha	19	19	1.00
Q7TNY6	ACBD3	acyl-Coenzyme A binding domain containing 3	7	7	1.00
Q9Z2I6	Sv2c	synaptic vesicle glycoprotein 2c	2	2	1.00
O08774	rgs12	regulator of G-protein signaling 12	2	2	1.00
Q5XHY7	STAM2	signal transducing adaptor molecule (SH3 domain and ITAM motif) 2	2	2	1.00
Q6P730	DAB2IP	DAB2 interacting protein	3	3	1.00
P47728	CALB2	calbindin 2	4	4	1.00
A8WCF8	TPRG1L	hypothetical protein LOC687090	4	4	1.00
Q04940	NRGN	neurogranin	4	4	1.00
P21818	STMN2	stathmin-like 2	7	7	1.00
P97546	NPTN	neuroplastin	13	13	1.00
*P62024	PHACTR1	phosphatase and actin regulator 1	6	6	1.00
Q8VH46	AFAP1	actin filament associated protein 1	2	2	1.00
Q9EPH2	MARCKSL1	MARCKS-like 1	5	5	1.00
Q80WD1	RTN4RL2	reticulon 4 receptor-like 2	5	5	1.00
Q9JLU4	SHANK3	SH3 and multiple ankyrin repeat domains 3	5	5.5	1.10
Q62717	CADPS	Ca++-dependent secretion activator	13.5	10	0.74
308571	LRRC4B	leucine rich repeat containing 4B	10.5	9	0.86
289820	ACTR2	ARP2 actin-related protein 2 homolog (yeast)	18	18	1.00
304543	MLEC	malectin	5	5	1.00
24915	PDLIM4	PDZ and LIM domain 4	4	4	1.00
292139	SHTN1	shootin 1	3	3	1.00
Q08163	CAP1	CAP, adenylate cyclase-associated protein 1 (yeast)	26.5	27	1.02
Q6P502	Cct3	chaperonin containing Tcp1, subunit 3 (gamma); similar to chaperonin containing TCP1, subunit 3 (gamma)	48	49	1.02
P35465	Pak1	p21 protein (Cdc42/Rac)-activated	4.5	3.5	0.78

Table 2. (*Continued)*					
Q62813	lsamp	limbic system-associated membrane protein	34	36.5	1.07
Q2HWF0	fnbp1l	formin binding protein 1-like	5	4	0.80
P70587	lrrc7	leucine rich repeat containing 7	9.5	14	1.47
O08719	EVL	Enah/Vasp-like	6.5	6.5	1.00
Q05140	SNAP91	synaptosomal-associated protein 91	28.5	30.5	1.07
100911769|59317	EPB41L1	erythrocyte membrane protein band 4.1-like 1	33	33.5	1.02
Q9Z2Q1	SEC31A	SEC31 homolog A (S. cerevisiae)	13	14	1.08
Q62847	ADD3	adducin 3 (gamma)	12.5	14	1.12
Q9WTP0	Epb4.1l1	erythrocyte protein band 4.1-like 1	33	33.5	1.02
Q91ZN1	Coro1a	coronin, actin binding protein 1A	3.5	4.5	1.29
Q9Z269	vapB	VAMP (vesicle-associated membrane protein)-associated protein B and C	17	19	1.12
64159	SPTAN1	spectrin alpha, non-erythrocytic 1	319	341	1.07
P16086	Spna2	alpha-spectrin 2	319	341	1.07
P61265	STX1B	syntaxin 1B	24.5	22	0.90
29138	BSN	bassoon presynaptic cytomatrix protein	34	25.5	0.75
308869|100361543	LAMTOR1	late endosomal/lysosomal adaptor, MAPK and MTOR activator 1	2.5	3	1.20
297699	STRAP	serine/threonine kinase receptor associated protein	8	9	1.13
Q9WU70	STXBP5	syntaxin binding protein 5 (tomosyn)	3	3.5	1.17
P48679	lmna	lamin A	14	20	1.43
Q9WV48	SHANK1	SH3 and multiple ankyrin repeat domains 1	41	47	1.15
306183	FARP1	FERM, ARH/RhoGEF and pleckstrin domain protein 1	9.5	10	1.05
Q9JK11	rtn4	reticulon 4	27	28	1.04
Q920Q0	palm	paralemmin	25	31	1.24
Q99N37	arhgap17	Rho GTPase activating protein 17	4	5	1.25
Q66H80	Arcn1	archain 1	2.5	2	0.80
Q66HR2	MAPRE1	microtubule-associated protein, RP/EB family, member 1; similar to Microtubule-associated protein RP/EB family member 1 (APC-binding protein EB1) (End-binding protein 1) (EB1)	35	44	1.26
Q9QUH6	SYNGAP1	synaptic Ras GTPase activating protein 1 homolog (rat)	30	39.5	1.32
O35889	mllt4	myeloid/lymphoid or mixed-lineage leukemia	13	17.5	1.35

Table 2. (*Continued)*					
P17077	rpl9	similar to hypothetical protein; hypothetical gene supported by X51706; similar to ribosomal protein L9; similar to 60S ribosomal protein L9; ribosomal protein L9; EH-domain containing 2	6	3	0.50
*Q63028	Add1	adducin 1 (alpha)	39.5	40.5	1.03
P31000	VIM	vimentin	60.5	64.5	1.07
361833	ANK3	ankyrin 3, node of Ranvier (ankyrin G)	24.5	35.5	1.45
P0C6S7	ANKS1B	ankyrin repeat and sterile alpha motif domain containing 1B	8.5	11.5	1.35
P08082	Cltb	clathrin, light chain (Lcb)	20	27	1.35
Q6XVN8	map1lc3a	microtubule-associated protein 1 light chain 3 alpha	6	7	1.17
O70441	SYN3	synapsin III	12	15	1.25
P55161	NCKAP1	NCK-associated protein 1	12.5	17	1.36
286930	DLGAP4	discs, large (Drosophila) homolog-associated protein 4	4.5	7.5	1.67
P63045	vamp2	vesicle-associated membrane protein 2	14	20	1.43
P15205	MAP1B	microtubule-associated protein 1B	86	122	1.42
290823	ERLIN2	ER lipid raft associated 2	7	10	1.43
**P19332	mapt	microtubule-associated protein tau	55.5	61.5	1.11
Q9Z250	Lin7a	lin-7 homolog a (C. elegans)	10	15	1.50
116493	GRIPAP1	GRIP1 associated protein 1	4	4	1.00
362173	CAPRIN1	cell cycle associated protein 1	44	22	0.50
**P97837	DLGAP2	discs, large (Drosophila) homolog-associated protein 2	3.5	7	2.00
Q9QX74	SHANK2	SH3 and multiple ankyrin repeat domains 2	15.5	31	2.00
P08592	APP	amyloid beta (A4) precursor protein	1	3	3.00
Q91XU1	qk	quaking	-3	-5	1.67
Q3MID3	ARFGAP 2	ADP-ribosylation factor GTPase activating protein 2	3	5	1.67
P24587	AKAP5	A kinase (PRKA) anchor protein 5	3	8	2.67
Q9WVE9	ITSN1	intersectin 1 (SH3 domain protein)	14	28.5	2.04
O35763	msn	moesin	6	9.5	1.58
Q8R4E1	ITFG1	integrin alpha FG-GAP repeat containing 1	4	7	1.75
*P97838	DLGAP3	discs, large (Drosophila) homolog-associated protein 3	3	4	1.33
P52481	CAP2	CAP, adenylate cyclase-associated protein, 2 (yeast)	11	6	0.55

Table 2. (*Continued)*					
O35867	ppp1r9a	protein phosphatase 1, regulatory (inhibitor) subunit 9A	18	10	0.56
114028	CTNND2	catenin delta 2	8	5	0.63
P08081	CLTA	clathrin, light chain (Lca)	13	19	1.46
Q9R080	gpsm1	G-protein signaling modulator 1 (AGS3-like, C. elegans)	6.5	9.5	1.46
65138	ADRM1	adhesion regulating molecule 1	1	3	3.00
114901	SORBS2	sorbin and SH3 domain containing 2	17	33	1.94
117106	SCARB2	scavenger receptor class B member 2	2	4	2.00
Q5FVH4	AKTIP	AKT interacting protein	1.5	2.5	1.67
**Q9Z327	SYNPO	myozenin 3; similar to myozenin 3; synaptopodin	19	39	2.05
338401	Crip2	cysteine rich protein 2	2.5	5.5	2.20
245709	EXOC8	exocyst complex component 8	10	9	0.90
Q9JIR4	RIMS1	regulating synaptic membrane exocytosis 1	16	11	0.69
P53563	BCL2L1	similar to Bcl2-like 1 isoform 3; Bcl2-like 1	1	3	3.00
P0C219	SLMAP	sarcolemma associated protein	2	7	3.50
298024	AKAP2	A-kinase anchoring protein 2	3	1	0.33
140592	Ppfia4	protein tyrosine phosphatase, receptor type, f polypeptide (PTPRF), interacting protein (liprin), alpha 4	1	4	4.00
Q05764	add2	adducin 2 (beta)	24.5	19	0.78
Q9QZM5	Abi1	abl-interactor 1	6	7.5	1.25
Peptidase					
366894	TRHDE	thyrotropin-releasing hormone degrading enzyme	5	1	0.20
P55213	Casp3	caspase 3, apoptosis related cysteine protease	6	4	0.67
O09175	Rnpep	arginyl aminopeptidase (aminopeptidase B)	11.5	10.5	0.91
Q07009	CAPN2	calpain 2	4.5	4	0.89
Q64537	capns1	calpain, small subunit 1	2	3	1.50
P00787	CTSB	cathepsin B	8	4	0.50
Q9JHW1	cpd	carboxypeptidase D	2	5	2.50
Phosphatase					
P20650	PPM1A	protein phosphatase 1A, magnesium dependent, alpha isoform	4	1	0.25
727679	PDXP	pyridoxal (pyridoxine, vitamin B6) phosphatase	9	3	0.33
P08289	ALPL	alkaline phosphatase,	4	2	0.50

Table 2. (*Continued)*					
Q64604	ptprf	protein tyrosine phosphatase, receptor type, F	28	26	0.93
P97710	Sirpa	signal-regulatory protein alpha	23	14	0.61
25613	PTPRZ1	protein tyrosine phosphatase, receptor type Z1	18.5	10	0.54
315648	PPP2R1B	protein phosphatase 2 regulatory subunit A, beta	10.5	5	0.48
25529	PTPRS	protein tyrosine phosphatase, receptor type S	46.5	38	0.82
Q7TMB7	LPPR4	plasticity related gene 1	17	17	1.00
29714	PTPRN2	protein tyrosine phosphatase, receptor type N2	4.5	4	0.89
24675	PPP3CB	protein phosphatase 3, catalytic subunit, beta isozyme	45.5	46	1.01
P63331	PPP2CA	protein phosphatase 2 (formerly 2A), catalytic subunit, alpha isoform	7.5	8.5	1.13
140591	PPFIA3	protein tyrosine phosphatase, receptor type, f polypeptide (PTPRF), interacting protein (liprin), alpha 3	40	27	0.68
Transcription regulator					
Q9QW30	Notch2	Notch homolog 2 (Drosophila)	3	1	0.33
P97879	GRIP1	glutamate receptor interacting protein 1	2	1	0.50
Q9Z1W6	MTDH	metadherin	4	3	0.75
P97603	NEO1	neogenin homolog 1 (chicken)	110	84	0.76
P58405	STRN3	striatin, calmodulin binding protein 3	5	4	0.80
Q9WU82	CTNNB1	catenin (cadherin associated protein), beta 1	31	25.5	0.82
P67779	Phb	similar to prohibitin; prohibitin	22	22.5	1.02
**Q9Z1P2	actn1	actinin, alpha 1	30	32	1.07
P18418	CALR	calreticulin	8	9.5	1.19
*Q05175	Basp1	brain abundant, membrane attached signal protein 1	30.5	31	1.02
Q6IRE4	Tsg101	tumor susceptibility gene 101	3	4	1.33
Q3T1J1	eif5a	eukaryotic translation initiation factor 5A	6.5	3	0.46
P38983	rpsA	similar to 40S ribosomal protein SA (p40) (34/67 kDa laminin receptor); ribosomal protein SA	14	13.5	0.96
Transmembrane receptor					
P29534	Vcam1	vascular cell adhesion molecule 1	5	1	0.20
P97829	CD47	Cd47 molecule	4	1	0.25
O55005	ROBO1	roundabout homolog 1 (Drosophila);	6	1.5	0.25

Table 2. (*Continued)*					
Q08406	CNTFR	ciliary neurotrophic factor receptor	2.5	1	0.40
P35053	GPC1	glypican 1	6.5	4.5	0.69
81005	NPTXR	neuronal pentraxin receptor	12	10	0.83
246331	NRP1	neuropilin 1	4	3	0.75
25311	DCC	DCC netrin 1 receptor	55	43	0.78
*Q99068	Lrpap1	low density lipoprotein receptor-related protein associated protein 1	15.5	16.5	1.06
Q9R066	cxadr	coxsackie virus and adenovirus receptor	13	11.5	0.88
309280	PLXNA3	plexin A3	3	7	2.33
291948	PGRMC1	progesterone receptor membrane component 1	5	11	2.20
Transporter					
Q62876	SYNGR1	synaptogyrin 1	4	1	0.25
Q6AY86	Vps26a	vacuolar protein sorting 26 homolog A (S. pombe)	3.5	1	0.29
Q62634	slc17a7	solute carrier family 17 (sodium-dependent inorganic phosphate cotransporter), member 7	6	2	0.33
P62744	AP2S1	adaptor-related protein complex 2, sigma 1 subunit	5	2	0.40
Q9JI51	vti1a	vesicle transport through interaction with t-SNAREs homolog 1A (yeast)	5	2	0.40
P31662	SLC6A17	solute carrier family 6 (neurotransmitter transporter), member 17	7	3	0.43
*Q4V887	SLC39A6	solute carrier family 39 (metal ion transporter), member 6	9	4	0.44
P53987	SLC16A1	solute carrier family 16, member 1 (monocarboxylic acid transporter 1)	4	2	0.50
Q63564	SV2B	synaptic vesicle glycoprotein 2b	6	3	0.50
Q07647	SLC2A3	solute carrier family 2 (facilitated glucose transporter), member 3	6.5	3.5	0.54
60391	NRXN1	neurexin 1	47	26.5	0.56
Q9R1N3	Slc4a7	solute carrier family 4, sodium bicarbonate cotransporter, member 7	7	4	0.57
P07825	Syp	synaptophysin	4	2.5	0.63
64832	CPLX1	complexin 1	11	7	0.64
*Q9JKS6	PCLO	piccolo (presynaptic cytomatrix protein)	16.5	9	0.55
O54922	EXOC7	exocyst complex component 7	3	2	0.67
Q99376	TFRC	transferrin receptor	7	4.5	0.64
P35952	Ldlr	low density lipoprotein receptor	13	9	0.69
**P21707	Syt1	synaptotagmin I	41	32	0.78

Table 2. (*Continued)*					
P07340	Atp1b1	ATPase, Na+/K+ transporting, beta 1 polypeptide	20	14.5	0.73
O35430	apbA1	amyloid beta (A4) precursor protein-binding, family A, member 1	11.5	8	0.70
P24942	SLC1A3	solute carrier family 1 (glial high affinity glutamate transporter), member 3	24	15.5	0.65
Q7TMA5	Apob	apolipoprotein B (including Ag(x) antigen)	4	3	0.75
Q794F9	SLC3A2	solute carrier family 3 (activators of dibasic and neutral amino acid transport), member 2	30.5	22	0.72
25696	VLDLR	very low density lipoprotein receptor	4	3	0.75
171147	SLC2A13	solute carrier family 2 (facilitated glucose transporter), member 13	4	3	0.75
P31596	Slc1a2	solute carrier family 1 (glial high affinity glutamate transporter), member 2	36	28.5	0.79
**Q63633	SLC12A5	solute carrier family 12 (potassium-chloride transporter), member 5	22	17.5	0.80
Q5U211	SNX3	similar to sorting nexin 3	5	4	0.80
Q6QIX3	SLC30A3	solute carrier family 30 (zinc transporter), member 3	5	4	0.80
Q64542	atp2b4	ATPase, Ca++ transporting, plasma membrane 4	49	42	0.86
P62944	AP2B1	adaptor-related protein complex 2, beta 1 subunit; similar to adaptor-related protein complex 2, beta 1 subunit	89	71	0.80
Q6PCU2	ATP6V1E1	ATPase, H+ transporting, lysosomal V1 subunit E1	17	14	0.82
Q66HR0	SLC12A9	solute carrier family 12 (potassium/chloride transporters), member 9	6	5	0.83
**P32851	STX1A	syntaxin 1A (brain)	36	30	0.83
P15999	Atp5a1	ATP synthase, H+ transporting, mitochondrial F1 complex, alpha subunit 1, cardiac muscle	79	67	0.85
P25286	ATP6V0A1	ATPase, H+ transporting, lysosomal V0 subunit A1	45.5	38.5	0.85
P97849	SLC27A1	solute carrier family 27 (fatty acid transporter), member 1	3	2.5	0.83
P85969	napB	N-ethylmaleimide-sensitive factor attachment protein, beta	13	11.5	0.88
P18484	Ap2a2	adaptor-related protein complex 2, alpha 2 subunit	83.5	75	0.90

Table 2. (*Continued)*					
P52303	AP1B1	adaptor-related protein complex 1, beta 1 subunit	50	42	0.84
P23978	SLC6A1	solute carrier family 6 (neurotransmitter transporter, GABA), member 1	4.5	4	0.89
116595	NRXN2	neurexin 2	46.5	29	0.62
Q641Z6	Atg2a	ATG2 autophagy related 2 homolog A (S. cerevisiae); EH-domain containing 1	16	15	0.94
O35142	copb2	coatomer protein complex, subunit beta 2 (beta prime)	1.5	3.5	2.33
287721	VAT1	vesicle amine transport 1	14.5	14	0.97
P11505	ATP2B1	ATPase, Ca++ transporting, plasma membrane 1	68.5	67	0.98
P11506	ATP2B2	ATPase, Ca++ transporting, plasma membrane 2	52	51.5	0.99
P61765	STXBP1	syntaxin binding protein 1	55.5	52.5	0.95
Q62991	SCFD1	sec1 family domain containing 1	4	4	1.00
Q5QD51	AKAP12	A kinase (PRKA) anchor protein 12	2	2	1.00
Q63616	Vps33B	vacuolar protein sorting 33 homolog B (yeast)	3	3	1.00
Q63344	slc16a7	solute carrier family 16, member 7 (monocarboxylic acid transporter 2)	3	3	1.00
P13638	Atp1b2	ATPase, Na+/K+ transporting, beta 2 polypeptide	3.5	3.5	1.00
Q99N27	SNX1	sorting nexin 1	4	4	1.00
25673	ANXA5	annexin A5	4	4	1.00
252881	EXOC3	exocyst complex component 3	4	4	1.00
Q02563	SV2A	synaptic vesicle glycoprotein 2a	10.5	11	1.05
P06686	Atp1a2	ATPase, Na+/K+ transporting, alpha 2 polypeptide	57.5	58.5	1.02
O70257	STX7	syntaxin 7	7	7	1.00
P06685	ATP1A1	ATPase, Na+/K+ transporting, alpha 1 polypeptide	83	87.5	1.05
**P06687	atp1a3	ATPase, Na+/K+ transporting, alpha 3 polypeptide	136	142	1.04
Q99MZ8	LASP1	LIM and SH3 protein 1	25	27.5	1.10
Q64568	Atp2b3	ATPase, Ca++ transporting, plasma membrane 3	32	35.5	1.11
Q63016	SLC7A5	solute carrier family 7 (cationic amino acid transporter, y+ system), member 5	9	9.5	1.06
P84092	AP2M1	adaptor-related protein complex 2, mu 1 subunit	39.5	44.5	1.13
Q9Z2I7	SVOP	SV2 related protein	6	7	1.17

Table 2. (*Continued)*					
P02650	APOE	apolipoprotein E	40	38	0.95
P10719	ATP5B	ATP synthase, H+ transporting, mitochondrial F1 complex, beta polypeptide; similar to ATP synthase beta chain, mitochondrial precursor	175.5	176.5	1.01
P26453	bsg	basigin	5	6	1.20
Q6AXS4	ATP6AP2	ATPase, H+ transporting, lysosomal accessory protein 2	20	24	1.20
P31647	SLC6A11	solute carrier family 6 (neurotransmitter transporter, GABA), member 11	7.5	7.5	1.00
Q32Q06	ap1m1	adaptor-related protein complex 1, mu 1 subunit	7.5	7	0.93
Q08851	STX5	syntaxin 5	4	5	1.25
100359512	VTI1B	vesicle transport through interaction with t-SNAREs 1B	4	5	1.25
Q4AEF8	CopG	coatomer protein complex, subunit gamma	7	9	1.29
P62815	atp6v1b2	ATPase, H transporting, lysosomal V1 subunit B2	46.5	56.5	1.22
83612	SLC32A1	solute carrier family 32 (GABA vesicular transporter), member 1	6	8	1.33
**P60881	SNAP25	synaptosomal-associated protein 25	15.5	21.5	1.39
Q5FVI6	ATP6V1C1	ATPase, H+ transporting, lysosomal V1 subunit C1	15.5	21.5	1.39
P54921	napA	N-ethylmaleimide-sensitive factor attachment protein, alpha	4	5.5	1.38
Q80ZA5	SLC4A10	solute carrier family 4, sodium bicarbonate transporter-like, member 10	2.5	3	1.20
P09951	syn1	synapsin I	28	40.5	1.45
Q06647	atp5o	ATP synthase, H+ transporting, mitochondrial F1 complex, O subunit	4	13	3.25
Q6RVG2	SLC4A8	solute carrier family 4 (anion exchanger), member 8	1	2	2.00
Q7TNK0	SERINC1	serine incorporator 1	1	2	2.00
Q01728	SLC8A1	solute carrier family 8 (sodium/calcium exchanger), member 1	5	10	2.00
P41542	USO1	USO1 homolog, vesicle docking protein (yeast)	4.5	3	0.67
P47709	RPH3A	rabphilin 3A	7	11	1.57
29501	SLC12A4	solute carrier family 12 (potassium/chloride transporter), member 4	1	3	3.00

Table 2. (*Continued)*					
P23514	COPB1	coatomer protein complex, subunit beta 1	8	3	0.38
P53678	AP3M2	adaptor-related protein complex 3, mu 2 subunit	2.5	7.5	3.00
Q9JI66	SLC4A4	solute carrier family 4 (anion exchanger), member 4	1	4	4.00

Note:
**: Proteins that are identified as synaptic candidate ubiquitinated proteins (Keil and Patrick 2010).*
***: Proteins that are identified as synaptic candidate ubiquitinated proteins and contain identified ubiquitination sites (Na and J.Peng 2012).*

Table 3: Synaptic candidate ubiquitinated membrane proteins			
Uniprot ID	**Gene Name**	**GG Peptides**	**GG sites**
P07632	superoxide dismutase 1, soluble	#N/A	#N/A
P11275	calcium/calmodulin-dependent protein kinase II alpha	K.DLINK#MLTINPSK.R	K250
P62024	phosphatase and actin regulator 1	#N/A	#N/A
P97838	discs, large (Drosophila) homolog-associated protein 3	#N/A	#N/A
Q62915	calcium/calmodulin-dependent serine protein kinase (MAGUK family)	R.ETGQQFAVK#IVDVAK.F	K41
P15791	calcium/calmodulin-dependent protein kinase II delta	K.IPTGQEYAAK#IINTK.K	K43
P59215	guanine nucleotide binding protein (G protein), alpha activating activity polypeptide O	R.AM%DTLGVEYGDK#ER.K	K98
Q9Z2L0	voltage-dependent anion channel 1	K.NVNAGGHK#LGLGLEFQA.-	K274
Q63028	adducin 1 (alpha)	#N/A	#N/A
Q9Z214	homer homolog 1 (Drosophila)	K.LTAALLESTANVK#QWK.Q	K221
Q9JKS6	piccolo (presynaptic cytomatrix protein)	#N/A	#N/A
Q9Z1P2	actinin, alpha 1	R.QK#ASIHEAWTDGK.E	K402
P32851	syntaxin 1A (brain)	K.LK#SIEQSIEQEEGLNR.S	K94
Q99068	low density lipoprotein receptor-related protein associated protein 1	#N/A	#N/A
P54290	calcium channel, voltage-dependent, alpha2/delta subunit 1	#N/A	#N/A
Q8K3M6	ELKS/RAB6-interacting/CAST family member 2	#N/A	#N/A
P13596	neural cell adhesion molecule 1	K.DESK#EPIVEVR.T	K785
P19332	microtubule-associated protein tau	R.LQTAPVPMPDLK#NVR.S	K565
Q63633	solute carrier family 12 (potassium-chloride transporter), member 5	K.K#DLTTFLYHLR.I	K896
Q9Z327	myozenin 3; similar to myozenin 3; synaptopodin	K.GQVVPANK#TGILEESMAR.R	K596
Q05175	brain abundant, membrane attached signal protein 1	#N/A	#N/A
P21707	synaptotagmin I	K.LTVVILEAK#NLK.K	K297
P60881	synaptosomal-associated protein 25	R.M%LQLVEESK#DAGIR.T	K40
P07936	growth associated protein 43	K.EK#DDAPVADGVEK.K	K69
Q63198	contactin 1	#N/A	#N/A

Table 3. (*Continued)*			
P97837	discs, large (Drosophila) homolog-associated protein 2	K.AVLVSK#AEELLK.S	K709
Q4V887	solute carrier family 39 (metal ion transporter), member 6	#N/A	#N/A
P97836	discs, large (Drosophila) homolog-associated protein 1	R.EVYQK#ASVNMDQAVVK.S	K300
P06687	ATPase, Na+/K+ transporting, alpha 3 polypeptide	R.AVFK#GGQDNIPVLK.R	K424

Accumulation of ubiquitin-conjugates at the synaptic membrane

Because the turnover of many membrane proteins occurs through covalent attachment of a single or short-chain ubiquitin at K63 and subsequent degradation by the lysosome, we examined whether lysosomal activity regulates ubiquitin conjugation of membrane proteins (Clague and Urbe 2010, Schwarz and Patrick 2012). We first observed the effect of lysosomal activity on the overall protein landscape by using Sypro Ruby staining. The synaptic fraction from cultured cortical neurons was isolated after 12 hours treatment with a lysosomal protease inhibitor, leupeptin (200uM), and a compartment acidification inhibitor, chloroquine (200uM); total protein visualization showed a small increase from control after 12 hours of lysosomal perturbation (Fig.1). Although this is not a dramatic increase in intensity, this increase can be observed with higher resolution imaging (Not shown). Next, to specifically look at the difference in ubiquitin-conjugates after treatment, eluted proteins were assayed for ubiquitin-conjugates by anti-ubiquitin immunoblot. Remarkably, ubiquitin conjugates in the synaptic membrane fraction were abundant under lysosomal inhibition (Fig. 2). No detectable ubiquitinated proteins were bound by avidin beads alone, indicating the specificity of the biotin-avidin affinity isolation. This finding supports ubiquitin-dependent lysosomal targeting of synaptic membrane proteins and a significant portion of membrane protein turnover is due to the targeting of membrane proteins for degradation by the lysosome. To further prove that the accumulation of ubiquitin-conjugates on the cell membrane is selective for lysosome-mediated degradation, a similar experiment was done but with inhibition of the proteasome, MG132. In contrast to the effect of lysosomal inhibition, inhibition of proteasome had no significant effect on membrane ubiquitin-

conjugates (Fig.3). This indicates that the degradation of many membrane proteins occurs via lysosomal and not proteasomal mechanisms.

These initial experiments provide strong evidence that a significant portion of membrane protein turnover is due to the ubiquitination and subsequent lysosomal degradation. This leads to my thesis project, which is to identify ubiquitinated membrane proteins by using mass spectrometry, and to assess the role of lysosome-mediated degradation of membrane proteins by looking at abundance after lysosomal perturbation. This proteomic study can provide a basic understanding of the molecular composition of membrane protein population under normal condition and lysosomal dysfunction condition with molecular abundance, and build a foundation for further studies on synaptic function and plasticity.

Lysosomal inhibition by leupeptin, bafilomycin A1, and chloroquine

Three lysosomal inhibitors are used: leupeptin, bafilomycin A1, and chloroquine. Leupeptin is a lysosomal protease inhibitor that can inhibit cysteine, serine, and threonine peptidases (Maeda, Kawamura et al. 1971). Bafilomycin A1 is a potent specific V-ATPase inhibitor and is thus able to abolish lysosomal acidification. It prevents maturation of autophagosomes into autolysosomes by inhibiting fusion between autophagosomes and lysosomes (Yamamoto, Tagawa et al. 1998). Chloroquine is a lysosomotropic agent widely used to neutralize lysosomal pH and block lysosomal degradation (Solomon and Lee 2009). Although leupeptin, bafilomycin A1, and chloroquine are three widely used drugs for lysosomal inhibition, little is known about the effect of each on the cell health, lysosomal activity, the ability to disrupt the

autophagy pathway, and the ability to de-acidify cell compartments. To investigating the effect of each inhibitor has on the cortical neurons, we performed a series of control experiments, so that we can ensure our observed effects on membrane proteins were attributable to a specific blockade of lysosomal enzymatic activity and de-acidification of cellular compartment, and not neurotoxicity.

Cell integrity after drug treatments

Before we further precede our experiments, we want to obtain an optimal treatment time point where the accumulation of membrane ubiquitin-conjugates reaches maximum while cell integrity is preserved. We treated cortical neurons with 200uM leupeptin and chloroquine for 12 hours and 24 hours, and predicted that ubiquitin conjugations accumulation should increase with inhibition time. We noticed after 12 hours, robust ubiquitin-conjugates were observed at the synaptic membrane. However, this effect was not seen after 24 hours (Fig 1). One possible explanation is that the neuronal cell health was compromised at 24 hours drug treatment. To monitor the neurotoxicity in response to drug treatments, immunocytochemistry for neuronal marker, microtubule-associated protein 2 (MAP2), was used followed by microscopic visualization to obtain representative cell images after treatment. After 12 hours treatment with 200uM leupeptin by itself, 200uM chloroquine by itself, and the combination of both, neurons appear healthy with widespread dendritic branches (Fig 4). On the contrary, same treatments but with 24 hours showed loss of dendrites, and cells were dying. This suggests that 12-hour treatment with both leupeptin and chloroquine at 200uM was able to perturb the lysosome and preserve cell integrity at the same time,

while inhibition after 12 hours accelerates decline and dysfunction, which increases a cell's vulnerability and triggers apoptosis.

Disruption of autophagy by lysosomal inhibitors

Many studies focus on the ubiquitin proteasome system and the endosome-lysosome system, as these are the two major degradation pathways in the cell; the autophagy pathway, on the other hand, is less mainstream. Autophagy is responsible for cytoplasmic molecules degradation similar to the ubiquitin proteasome system, but targets for long-lived or aggregated proteins and damaged organelles, and it fuses with lysosomes for final degradation (Mizushima 2007). Since lysosome is required for the autophagy pathway, we then examine the effect of lysosomal dysfunction on the autophagy pathway. To determine whether the autophagy pathway is disrupted, LC3-II, a protein that binds to the autophagosomal membrane that it is commonly used as a marker of autophagy, was examined in treated samples (Mizushima 2007). In total lysates of control and treated samples, bafilomycin and chloroquine treatments, even at a low dose (50nM and 25uM relatively), significantly increased LC3-II levels (Fig 5). Leupeptin, on the other hand, does not disrupt the autophagy pathway with high dose treatment (2000uM) and long hour inhibition (24h). These data indicate that chloroquine and bafilomycin are capable of affecting autophagosome content, but not leupeptin.

Inhibition of lysosomal function by lysosomal inhibitors

To further test out the effects of lysosomal inhibitors, we used LysoTracker and a cell lysate-based cathepsin enzyme activity assay to look at the acidity of lysosomes and the lysosomal enzymatic activity after treatments. As expected, both bafilomycin and

chloroquine are able to abolish lysosomal acidification at low concentration; treatment at various concentrations with either bafilomycin or chloroquine abolishes LysoTracker staining (Fig 6). Treatment with leupeptin at 400uM did not affect the acidity of the lysosome, as its LysoTracker staining was similar to control. Next, cathepsin B activity was accessed through cell lysate-based activity assay. As expected, leupeptin, which targets at the proteases in the lysosome, effectively inactivated cathepsin B activity at the concentration 200uM while chloroquine was ineffective to inhibit cathepsin B (Fig 2d). These results indicate that 12 hours treatment with 200uM leupeptin inhibited a significant amount of lysosomal enzymatic activity in cortical neurons.

In summary, the optimal concentration we decided to use for mass spectrometry experiment is 200uM leupeptin and 200uM chloroquine for 12 hours. Under this condition, lysosome protease activity is inhibited, the autophagic activity is disrupted, and lysosomal acidification is abolished while cell health is preserved.

Identifying cell membrane proteins by mass spectrometry

Many well-known cell surface receptors such as EGFRs, GABARs, AMPARs, depend on lysosomal degradation as their final terminal, we thus asked whether there are other membrane proteins directly modified by ubiquitin and sent to lysosomal degradation pathway (Goh and Sorkin 2013, Schwarz, Hall et al. 2010, Arancibia-Carcamo, Yuen et al. 2009). To answer this question, we used live-labeling biotinylation followed by synaptic fractionation to purify synaptic membrane protein population in cortical neurons (DIV 21). Eluted proteins were then precipitated, solubilized, digested

and then subjected to liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). Data analysis is done to MS dataset.

We detected about 1600 unique proteins with tandem mass spectrometry, including about 539 plasma membrane proteins, and 149 of them contain transmembrane region. Receptors and channels, most of the major PSD glutamate receptors such as NMDA receptors (NR1, NR2B), AMPA receptors (GluR1, GluR2, GluR3, GluR4), and metabotropic glutamate receptors (mGluR5, mGluR7) were identified by mass spectrometry. Although many proteins were found localized at the plasma membrane, the majority was localized at the intracellular organelles including ER, mitochondria, nucleus, Golgi and so on. This is not surprising since our preparation of crude membrane fractions were included a significant proportion of intracellular transmembrane proteins. Using the spectral abundance of each identified protein from control and treated samples, we calculated the normalized Leu/Ctrl spectral ratio for each identified protein. We considered identified membrane proteins with a Leu/Ctrl ratio greater than 2 to be upregulated and less than 0.5 to be downregulated. Out of these plasma membrane proteins, we found that 105 of them were upregulated, and 207 were downregulated, while 226 did not significantly change in protein level.

Gene ontology (GO) analysis performed using two bioinformatics programs: Database for Annotation, Visualization and Integrated Discovery (DAVID), and Ingenuity Pathway Analysis (IPA), on the biological functions executed by plasma membrane proteins only. The functions of plasma membrane proteins were of interest because receptors and antigens involved in cellular signaling are often located on the cell

surface. In our data, the majority of plasma membrane proteins were identified as ion channels and transporters, implicating the important role of these molecules in the cellular homeostasis.

Based on a mass spectrometry data analysis done previously by a former lab member, Jeff Keil, 29 plasma membrane proteins were identified as synaptic candidate ubiquitinated proteins. In his experiment, he isolated and identified novel synaptic ubiquitinated proteins using mutant mice expressing 6-histidine-tagged ubiquitin and wild-type mice. He extracted synaptosomal membrane fractions from both samples and performed nickel affinity chromatography. And the eluted proteins were sent to tandem mass spectrometry. These 29 proteins were synaptic candidate ubiquitinated membrane proteins. To further investigate these proteins, we used another data analysis produced by Junming Peng and his colleages. We were able to find ubiquitination sites for 18 of these synaptic ubiquitinated membrane proteins with diglycine peptides sequence.

Additional function analysis done on these plasma membrane proteins indicates that there were 60 cell adhesion molecules, 40 cellular homeostasis regulators, 90 cellular localization regulators, and 14 calcium ion transport regulators in the mass spectrometry data set and some were altered in protein level. This suggests that the ubiquitin-dependent lysosomal degradation pathway is important in maintaining synaptic functions. We also looked at genes that involve in synaptic transmission and plasticity. In fact, there were a number of molecules involving inhibitory synaptic transmission. Ubiquitin-dependent regulation of receptor trafficking and turnover has been shown to be important for inhibitory synaptic transmissions, such as GABAR, are directly ubiquitinated to regulate

their trafficking and turnover at synapse. Using the pathway analysis generated by Ingenuity pathway analysis on GABA receptor signaling pathway, we found that many regulators that involve in GABAR signaling pathway such as AP2S1, GABRA3, ADCY2, ADCY5, GABRA1, GABBR2, AP2B1, AP1B1, AP2A2, SLC6A1, GAD2, GABBR1, SLC6A11, GNAS, AP2M1, GABRB3, GPHN, MRAS, and some of them downregulated under lysosomal inhibited conditions. Further experiments are needed to search for an answer. This however provides some information shows the crucial role on both excitatory and inhibitory synaptic transmission by controlling postsynaptic receptors numbers.

III. Discussion

Ubiquitin-dependent endocytosis of neuronal membrane proteins is a key biological mechanism to control synaptic plasticity by altering the number of membrane proteins (Schwarz and Patrick 2012). After internalization, these short-chain or mono-ubiquitinated proteins are either being recycled or targeted for lysosomal degradation (Clague and Urbe 2010). Here, we investigated the role of this regulated endocytic-sorting pathway on the internalization of membrane proteins by looking at the change of overall membrane protein levels under lysosomal inhibition.

We tested many different combinations of lysosome inhibitors and examined their effects on the lysosomal protease activity, autophagy pathway, acidification, and cell integrity. Treatment with lysosome inhibitors 200uM leupeptin + 200uM chloroquine for 12 hours was the most effective in blocking the internalization of membrane proteins, and causing the accumulation of membrane proteins without affecting cell viability. Treating neurons with leupeptin along with chloroquine, which inhibits acidification of intracellular compartments, shows more robust ubiquitin-conjugates accumulation compared to treating with leupeptin by itself. This could mean two things: chloroquine is able to completely abolish the endosome-lysosome pathway by deterring acidification, and thus, generate a bigger effect. It is also possible that the internalization of membrane proteins relates not only on the endosome-lysosome pathway, but also the autophagy pathway. Chloroquine is shown to have a strong effect on inhibiting the autophagy pathway; the autophagy pathway targets cytoplasmic molecules, such as damaged organelles and protein aggregates. It is possible that proteins that mediate membrane protein ubiquitination and internalization are degraded or regulated by the autophagy

pathway in the cytosol, and thus, the autophagy pathway could play a role in the internalization of membrane proteins indirectly.

In our initial data, an increased of ubiquitinated proteins was observed after inhibition of lysosomal activity. Was it caused by the accumulation of ubiquitinated proteins on the cell membrane or was it caused by an increased of ubiquitination of membrane proteins after inhibiting lysosomes? Could they become highly ubiquitinated after blocking lysosomal activity? If the proteasome is upregulated to compensate lysosomal dysfunction, it is possible that membrane proteins are being poly-ubiquitinated under lysosomal inhibition, and thus more likely to be sent for proteasomal degradation. This could be a compensatory mechanism in order to control the turnover of the membrane proteins under lysosomal dysfunction. Moreover, it has been thought that the ubiquitin proteasome system and the autophagy pathway are two independent pathways with no intersection. However, recent studies show that they can act as compensatory degradation system to each other (Pandey, Nie et al. 2007, Korolchuk, Mansilla et al. 2009, Kraft, Peter et al. 2010, Lamark and Johansen 2010). If the autophagic machinery and the proteasome were interconnected, the autophagy was inhibited by chloroquine, and proteasomal activity could be upregulated and caused membrane proteins poly-ubiquitinated. What is the proteasome activity under inhibition of lysosome and autophagy? Could lysosomal inhibition cause an increase in proteasomal activity?

Despite our mass spectrometry results did not show a robust increase in extracted membrane proteins after inhibition of lysosomal activity, we were able to identify molecular composition of overall extracted membrane proteins in the cortical neurons.

Most of the detected membrane proteins show a downregulation after lysosomal inhibition. Even though these results do not match with our expectation, it does not necessary mean there are not accumulation of membrane protein. In fact, there are more thorough ways to analyze the MS data, such as the topological network approach. This approach focuses on identifying the common regulators of a set of proteins based on their expression level. It locates upstream regulators of these proteins individually and elucidates the common regulators for a combined signaling pathway. Network topology of protein co-abundance networks identifies proteins that have been dismissed during measurement techniques or may not be highly regulated, and it allows for better predication of biologically important targets (McDermott, Diamond et al. 2012, Haider and Pal 2013).

There are many factors to consider for data improvement, such as the stability of ubiquitinated proteins in our prep condition, the effectiveness of our pulldown assay, and the data analyzing method. A major concern for biochemically purified synaptic membrane population is the contamination created by the subcellular structures, and it usually gives out low amount of total proteins. Such impurities would contribute artifactually to the observed multiplicity of proteins in the membrane population. The mass spectrometer is a concentration sensitive detector and yet, we failed to obtain a number of known, short-lived UPS regulators, and ubiquitin-related proteins. Many of these proteins are so quickly degraded after ubiquitination that they can be measured only after stabilization techniques. We used DUBs inhibitor 25mM N-Ethylmaleimide (NEM) to obtain ubiquitinated proteins. Is this sufficient to maintain ubiquitinated proteins?

Could it be possible that the number of purified ubiquitin-related proteins is small compared to the overall pulldown and make it insignificant in MS screening?

The ultimate goal of this project was to identify membrane substrates for ubiquitin conjugation and potential ligases of specific ubiquitinated proteins, I have tried to immunoprecipitate ubiquitin-conjugates using ubiquitin antibody in the membrane population by incorporating biotinylation in the experiment; however, I was not able to get it to work despite countless of attempts. Another alternative approach was to purify membrane proteins and compare the changes under lysosomal inhibition. However, the sensitivity of this mass spectrometry is not high and the detected membrane proteins are not all ubiquitin conjugates. The same experiment is worth repeating with the use of di-glycine antibody to enrich ubiquitinated proteins from the pull down population, with the goal of characterizing both ubiquitin conjugates and precise sites of ubiquitination.

IV. Materials and Methods

Antibodies and reagents.

Antibodies were as follows: mAb ubiquitin (P4D1; Santa Cruz Biotechnology); pAb LC3/MAP1LC3B (Novus); mAb Tubulin (Sigma); pAb MAP2 Chicken (Abcam). Reagents were as follows: Chloroquine diphosphate (CQ, MP Biomedicals); bafilomycin A1 (Baf A1, Fisher Scientific); leupeptin (Leup, Millipore); N-ethylmaleimide (NEM, Fisher Scientific); LysoTracker Red DND-99(Life Technologies); Cathepsin substrate Z-RR-AMC for cathepsin B (VWR International).

Neuronal cultures.

Rat dissociated hippocampal or cortical neurons from postnatal day 1 were plated at a density of 45,000 cells/cm2 onto poly-D-lysine-coated coverslips, 25 mm dishes (hippocampal cultures) (Mattek) or poly-D-lysine-coated 6-well plastic dishes, 10cm dishes (cortical cultures) and were maintained in B27 supplemented Neurobasal media (Invitrogen) until in vitro (DIV) 14-21, as described previously (Djakovic et al., 2009; Schwarz et al., 2010; Djakovic et al., 2012).

Biotinylation of surface proteins.

Dissociated cortical neurons (DIV 21) were treated with leupeptin (200uM) and chloroquine (200uM) for 12 hours prior to biotinylation. To purify surface membrane proteins, cultured cortical neurons were rinsed with PBS-MC (10 mM phosphate buffer, 2.7 mM KCl, 137 mM, NaCl, 1 mM CaCl2, 0.5 mM MgCl2, 25mM NEM, pH 7.4), placed on ice, rinsed with cold PBS-MC, incubated with 0.5mg/ml Sulfo-NHS-SS-Biotin (Pierce) for 30 min at 4°C, and then rinsed again with 0.1% BSA in PBS-MC. Cells were

scraped into HEPES-buffered sucrose (0.32 M sucrose, 4 mM HEPES, 25mM NEM, pH 7.4, supplemented with protease inhibitors) and performed synaptic membrane enrichment. Protein concentration of synaptic membrane fraction was then measured by BCA protein assay (Pierce), and an equal amount of protein per sample was incubated with monomeric avidin agarose (Pierce) overnight at 4°C. Agarose was then rinsed 3 times, and bound proteins were stored in HEPES-buffered sucrose and were used for LC-MS/MS analysis.

Synaptosome isolation.

Rat cortical neurons were homogenized in HEPES-buffered sucrose (0.32 M sucrose, 4 mM HEPES, pH 7.4, 25mM NEM, and protease inhibitors) with 15 strokes in a glass Teflon homogenizer. The homogenate was centrifuged at 1,000 X g for 10 min at 4 °C to remove nuclei and cellular debris (P1). The supernatant (S1) was centrifuged at 15,000 X g for 15 min at 4°C to yield a synaptosomal pellet (P2). The resulting pellet was suspended with 1 ml HEPES-buffered sucrose solution and was used for subsequent pull-down experiment.

Mass spectrometry and identification of peptides and proteins.

Purified synaptic membrane samples were digested sequentially with trypsin. Eluted peptides were analyzed by LC-MS/MS on an LTQ Orbitrap Velos. MS/MS spectra searches (Sequest), target-decoy peptide filtering, and linear discriminant analysis were performed as described (Huttlin et al., 2010) with an initial 1% peptide level false discovery rate and final protein level false discovery rate of 1%.

Statistical analysis

The fold change was transformed using the log2 function, so that the data is centered around zero.

Total protein visualization

Cell membrane proteins of Synaptic fraction were separated by SDS-PAGE and visualized by staining fixed gels with Sypro Ruby.

SDS-PAGE and western blot.

Total protein lysates were generated by scraping cells into RIPA buffer with protease inhibitors, incubating for 20 min at 4°C, and centrifuging at 14,000 rpm at 4°C. Protein concentration was determined by BCA protein assay (Pierce), and equal protein amounts were loaded. Samples were boiled with sample buffer, resolved on 12% SDS-PAGE, and probed with primary and then secondary HRP-conjugated antibodies. Blots were digitized by scanning films.

Estimation of intralysosomal pH using LysoTracker.

The intralysosomal pH was estimated using LysoTracker, following manufacturer's instructions. The fluorescence intensity was observed under a confocal microscope (Leica DMI6000 inverted microscope).

Cathepsin B activity assay.

The cathepsin B enzymatic activity was measured using a cell lysate-based assay that has already been established (Zhou J et al. 2012). Briefly, cells were lysed in M2 buffer, and different concentration of drugs were added to the lysate and then incubated with 50uM of the fluorogenic cathepsin B substrate Z-RR-AMC in 100ul cell-free system buffer (10mM HEPES-NaOH, pH 7.4, 220mM mannitol, 68mM sucrose, 2mM NaCl, 2.5 mM KH2PO4, 0.5 mM EGTA, 2mM MgCl2, 5 mM pyruvate, 0.1 mM PMSF and 1 mM dithiothreitol) in a 96-well plate for 1h at 37°C. The fluorescence intensity was monitored by a fluorometer at an excitation wavelength of 380 nm and an emission wavelength of 460 nm.

Immunostaining.

After drug treatment, neurons were washed with cold PBS-MC and fixed with a 4% PFA/sucrose solution for 10 min. Cells were permeabilized with 0.2% Triton X-100 and 2% BSA in PBS-MC for 20 min, followed by overnight block in 5% BSA in PBS-MC. Primary and secondary antibodies were diluted into 2% BSA in PBS-MC and applied to neurons, overnight at 4°C for primary and 1h at room temperature for secondary. Coverslips were mounted onto glass slides for confocal imaging.

Confocal microscopy and image analysis.

All images were acquired with a Leica DMI6000 inverted microscope equipped with a Yokogawa Nikon spinning I6000 inverted microscope equipped with a Yokogawa Nikon spinning disk confocal head, an Orca ER high-resolution black and white cooled CCD camera (6.45 m/pixel at 1), Plan Apochromat 63/1.4 numerical aperture objective,

and an argon/krypton 100 mW air-cooled laser for 488/568/647 nm excitations. Maximum projected Z-stacks were analyzed with National Institutes of Health ImageJ.

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