Design and optimization of small peptides that regulate the balance of synaptic excitation and inhibition

by

Xi Chen

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Abstract

The balance of synaptic excitation and inhibition plays a very important role in maintaining the function of central nervous system (CNS) and the imbalance is involved in neurologic diseases such as autism and epilepsy. PSD95 and gephyrin have been studied as scaffolding proteins, having critical functional and structural roles in excitatory and inhibitory synapses, respectively. In the thesis, I have attempted to develop small systemically applicable peptides that can reversibly knock down PSD95 or gephyrin in vitro and in vivo, using the novel peptide-mediated technology recently developed in our lab, as tools for modulating the balance of synaptic excitation and inhibition. The efficacy of the peptides knocking down respective targeted proteins was tested by immunoblots after the cultured neurons were treated with the peptides for the desired time at various concentrations. I found that peptides that target either PSD95 or gephyrin showed toxicity to the neurons in a dose and time dependent manner utilizing the LDH assay. The toxicity may also contribute to the reduction of protein levels. Using one of the peptides, TAT-NR2B9C-CMA that targets PSD95 as example, I systemically investigated the causes of the toxicity and tested several strategies to reduce the toxicity while keeping the efficacy of the protein knockdown. I found that while multiple treatments at low dose could not successfully separate the cell death and knockdown effect, treatment at high doses with shortened durations appeared partially effective in reducing the toxicity and maintaining knockdown efficacy. However, this protocol may not be applicable in vivo. I next modified the intrinsic properties of peptides by shortening CMA targeting motifs and/or adding a linker between the binding sequences and CMA targeting motif. I found that while both strategies could decrease the toxicity with varied degree, peptides with short CMA targeting motif kept the

knockdown efficacy. Taken together, my study demonstrated the effective strategies to reduce the toxicity of the peptides one can consider in the process of developing novel protein knockdown peptides as novel research tools and therapeutic reagents.

Preface

This thesis is based on the work conducted by Dr. Yu Tian Wang and Xi Chen in Brain Research Centre in University of British Columbia. Dr. Yu Tian Wang and I identified and designed the project. The peptides were synthesized in Dr. Yu Tian's lab by a group led by Dr. Lidong Liu. I was responsible for conducting all the experiments and performing all data analysis under the supervision of Dr. Yu Tian Wang.

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List of Abbreviations

CNS	Central nervous system
GABA	Gamma-amino butyric acid
NMDAR	N-Methyl-D-aspartic acid receptors
AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors
PSD	Postsynaptic density
MAGUK	Membrane associated guanylate kinases
SH3	src homology 3
GK	guanylate kinase
nNOS	neuronal nitric oxide synthase
GlyR	Glycine receptor
GephE	Gephyrin E domain
MEF2	myocyte enhancer factor 2
FMRP	fragile X mental retardation protein
LMP	lysosome membrane proteins
CMA	Chaperone-mediated autophagy
CTM	CMA targeting motif
CPP	cell penetrating peptide
TAT	trans-activating transcriptional activator
HIV 1	human immunodeficiency virus 1
SPPS	solid-phase peptide synthesis

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Dedication

Dedicated to my mom

Chapter 1: Introduction

1.1 Overview

A fine balance between excitatory and inhibitory synaptic transmissions (E-I balance) is the key to maintain the normal level of neuronal activity. Imbalance between excitatory and inhibitory synaptic transmissions would cause dysfunctions, contributing to the pathogenesis of numerous brain diseases. As such, developing effective means to modulate/fine-tune the E-I balance may lead to effective therapeutics for treating many brain diseases whose pathogenesis involve disrupted E-I balance. Different methods have been applied to modulate the E-I balance either at DNA, RNA or protein levels based on the "central dogma". In this dissertation, I have attempted to develop a novel means to regulate the E-I balance via controlling the levels of PSD95 (a scaffolding protein required for the formation of excitatory synapses) or gephyrin (a scaffolding protein required for inhibitory synapses) with a newly developed peptide technology, peptide-mediated protein knockdown. Using this technology, I have developed and characterized peptides that specifically knock down PSD95 or gephyrin. In addition, I have also applied various strategies to optimize these peptides so as to make them work more efficiently with reduced cytotoxicity. The introduction will contain two main topics: regulation of the excitatory and inhibitory balance in central nervous system (CNS) with a particular emphasis on roles of PSD95 and gephyrin, and the newly developed technology of small peptide-mediated degradation.

1.2 Excitatory and inhibitory balance in CNS

Balance of excitation and inhibition refers to the relative contributions of excitatory and inhibitory synapses corresponding to neuronal events. It plays a vital role in the normal function of central nervous system. Excitatory synaptic transmission in the brain is mainly mediated by the neurotransmitter glutamate while the inhibitory synaptic transmission is mainly mediated by gamma-amino butyric acid (GABA) and glycine. And those neurotransmitters and their receptors interact closely with the scaffolding proteins which are involved in the coordinated regulation of excitatory and inhibitory balance.

1.2.1 Role of PSD95 in excitatory synapses

Synapses are considered as excitatory when the possibility of firing an action potential is increased and glutamate is the transmitter that mediates synaptic transmission at most excitatory synapses in the CNS. The most common ion channels that are activated by glutamate are N-Methyl-D-aspartic acid receptors (NMDAR), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPAR) and kainate receptors. Another big group activated by glutamate is metabotropic receptors which are coupled to G proteins.

Excitatory synapses contain an electron-dense region, referred to as the postsynaptic density (PSD), among which PSD95 is the most abundant and best characterized component (Kim E et al 2004). PSD95 belongs to the family of membrane associated guanylate kinases (MAGUKs), and the neuronal MAGUKs also include PSD93, SAP102 and SAP97 (Elias et al 2008). PSD95 organizes the architecture by its interactions with many other proteins. PSD95 was found to bind directly with C terminal of NMDA subunit which is common to all GluN2 subunits by two-yeast

hybrid (Kornau et al 1995, Niethammer et al 1996). And for the AMPARs, PSD95 was found to bind with AMPARs via Stargin/TARP (Elias & Nicoll 2007). PSD95 interacts with many other proteins such as nNOS, Citron, and neuroligin (Chen et al 2011), making it very important in the organization of PSD and relaying second-message signaling transduction at the excitatory synapses.

Many lines of evidence have shown the importance of PSD95 to excitatory synapses by its overexpression and knockdown or knock out in mice. Overexpression of PSD95 in hippocampal neurons drove maturation of glutamatergic synapses (El-Husseini et al 2000). Knockdown of PSD95 using antisense oligonucleotides induced significant cell death in both hippocampal culture and slices within 24h (Gardoni et al 2002). Blocking expression of endogenous PSD95 by transfection of siRNA resulted in an increase in inhibitory synapses and a decrease in excitatory synapses while the total number of synaptic contacts showed no change (Prange et al 2004). Although PSD proteins are widely expressed in the central nervous system, the relative abundance of PSD in specific brain regions varies a lot at different stages of development (Zheng et al 2012). In the late embryonic and early postnatal stages, expression of SAP102 is high and decreases with time in hippocampus. Expression of PSD95 is quite low in the early postnatal stage and increases as the mouse ages (Elias & Nicoll 2007, Sans et al 2000). The modulation of PSD95 at post translational level has to be done postnatally due to its expression pattern in development.

PSD95 is composed of 3 PDZ domains, a src homology 3 (SH3) domain and a guanylate kinase (GK) domain. An intramolecular interaction between the SH3 domain and GK domain is

observed, but the function of this interaction is unclear (Tavares et al 2001). PDZ domains are modules that regulate protein interactions at the extreme carboxyl (C) terminal and/or internal of other proteins. The canonical PDZ domains contain 80-100 amino acids and form a globular structure consisting of a six stranded β-barrel flanked by two α-helices (Tochio et al 2000). Among the three PDZ domains in PSD95, the second is well studied. PDZ2 was found to bind with the nine amino acids (KLSSIESDV) from C terminal of NR2B subunit (Kornau et al 1995, Niethammer et al 1996). Later, this PDZ2 domain was also found to interact with the internal sequence of neuronal nitric oxide synthase (nNOS), which made PSD95 a target to reduce excitotoxicity by blocking the downstream pathways. The nine amino acid peptide NR2B9C has been developed to perturb NMDAR and PSD95 interaction, thus protecting neurons from excitotoxicity and ischemia (Aarts et al 2002, Cook et al 2012, D'Mello et al 2011). Among so many PDZ domains in human, the PDZ2 of PSD95 has the strongest binding with NR2A-NR2C subunits (EC_{50}, ~1uM) and fusing NR2B9C to TAT enhances its affinity to PDZ2 by 100-fold (EC_{50}, ~7nM) (Cui et al 2007). Due to the high affinity of binding between TAT-NR2B9C and PSD95, TAT-NR2B9C is now in the phase III clinical trial to treat stroke. Taking together, PSD95 is an efficient target to modulate the excitatory synapses.

1.2.2 Role of gephyrin in inhibitory synapses

Synapses are considered as inhibitory when the possibility of firing an action potential is decreased. GABAergic and glycinergic synapses are the main inhibitory synapses in CNS. In the adult CNS, activation of GlyR and $GABA_AR$ leads to the increased conductance of chloride which result in hyperpolarization and inhibition of postsynaptic neurons(Betz & Laube 2006). GABAergic transmission almost affects every neuron in the CNS, while glycinergic transmission

mainly works in caudal CNS structure (Dutertre et al 2012). Similar to PSD95, gephyrin functions at the clustering of glycine receptors and GABAA receptors at inhibitory synapses but to a different extent (Fritschy et al 2008, Kneussel et al 2001, Levi et al 2004, Maas et al 2006). Besides its role in the organization of inhibitory synapses, gephyrin is also responsible for molybdenum cofactor synthesis in metabolism. Gephyrin is abundant in liver, kidney and other non-neuronal organs where it plays the role of enzyme (Nawrotzki et al 2012).

Glycinergic synapses mediate fast inhibitory neurotransmission mainly in the spinal cord, brainstem and caudal brain (Dutertre et al 2012). The GlyR was the first neurotransmitter receptor to be purified from mammalian CNS (Laube 2002). It belongs to the Cys loop superfamily of ligand-gated ion channels. The subunit stoichiometry of GlyR was originally thought to be 3α:2β (Burzomato et al 2003, Langosch et al 1988), but later was revised to 2α:3β using co-expression of wild-type α or βsubunits with α/β tandem construct (Grudzinska et al 2005). So far, four different genes called Glrα 1-4 have been identified to encode α subunits while only one gene Glrβ has been identified to encode β subunits in vertebrates (Lynch 2004). The expression of Glrα 1-4 is regulated in a temporal and spatial way revealed by biochemical and in situ hybridization studies. GlyR α1 mRNA and protein is abundant in spinal cord, brain stem while α2 mRNA and protein are abundant at birth but decrease a lot in cortex and hippocampus when reaching adult. And α3 is detected at moderate level in spinal cord, cerebellum and olfactory bulb and α4 is almost undetectable in adult CNS. For GlyR β mRNA, it is expressed widely in CNS in both embryonic and postnatal stages (Harvey et al 2000, Malosio et al 1991, Sato et al 1992). The distribution of GlyRs determines that they mainly function in

spinal cord and brainstem as the motor and sensory neurons. GlyRs are also found in hippocampus and are involved in neuronal development and other neuronal activities.

The $GABA_AR$ is the principle inhibitory receptor in CNS (Macdonald & Olsen 1994). Similar to GlyR, it also belongs to the Cys-loop superfamily. Mammalian $GABA_ARs$ are heteropentameric channels that conduct chloride which are constructed from a combination of many possible subunits. Among those subunits, most endogenous $GABA_ARs$ in the brain are composed of 2α subunits, 2β subunits and 1γ subunit (Olsen & Sieghart 2009). The localization of $GABA_AR$ in both extracellular and cellular level largely depends on the subunit composition and the expression profile also changes with age. At postsynaptic sites, $GABA_AR$ composed of α (1-3) subunits, with β and γ subunits are localized (Smart & Paoletti 2012) while at extrasynaptic sites, $GABA_ARs$ are mainly composed of α5βγ and α (4 or 6)βδ subunits (Brickley & Mody 2012). Similar with the detection of localization of GlyR, immunohistochemistry and *in situ* hybridization are the main methods to depict the expression of $GABA_AR$ (Khrestchatisky et al 1991, Pirker 2000, Wisden et al 1992). In adult rat brain, the most abundant subunits α1, β2 and γ2 are detected in almost every brain region. α2 is widely expressed except for midbrain, thalamus and cerebellum and α3 is restricted to neocortex and hippocampus and claustrum of basal nuclei. α4 subunits are enriched in extrasynaptic sites in neocortex and hippocampus. The α5 is also found in hippocampus and neocortex while α6 is restricted to cerebellar granule cells. The β1 subunit is enriched in hippocampus and amygdala and β2 and β3 are found in most brain regions. The γ1 is detected in hippocampus, amygdala and hypothalamus and γ3 is in neocortex and basal nuclei. Other subunits such as ε and θ are not listed here (Khrestchatisky et al 1991,

Pirker 2000, Wisden et al 1992). The wide expression of $GABA_AR$ in brain regions is the basis for its role as the major inhibitory transmission.

The inhibitory GlyR synapses were found to depend on gephyrin clustering. When the GlyR was purified from spinal cord on aminostrychnine-agarose, three polypeptides were seen: the GlyRα, the GlyRβ and the cytosolic scaffolding protein gephyrin (Pfeiffer et al 1982). When the gephyrin-binding motif on GlyR is inserted into other proteins, it is sufficient to cause co-clustering with gephyrin (Tretter et al 2012). The interaction of Glyβ subunits and gephyrin is well established. The interactions between gephyrin and $GABA_A$ receptors are more complex. Gephyrin knock out mice die early postnatally and display loss of postsynaptic $GABA_ARs$ containing α2 and β2 subunits and GlyRs clustering, whereas glutamate receptor subunits were normally localized (Chen et al 2014). However, certain $GABA_AR$ subtypes still cluster in neurons lacking gephyrin either by knocking out gephyrin or by knocking down gephyrin using siRNA (Kneussel et al 2001, Levi et al 2004). It appears that gephyrin could only cluster part of the $GABA_ARs$-mainly α and β subunits to mediate inhibition.

Gephyrin contains three domains, 20kDa G domain in N-terminal, 43kDa E domain in C terminal and a large linker domain in between (Fritschy et al 2008, Maric et al 2014b). Gephyrin is quite conserved across different species. Different from PSD95, gephyrin does not contain PDZ domains but forms aggregates by auto-oligomerization. The isolated G domain forms stable trimers and the isolated E domain forms dimers in solution. The trimerization and dimerization may form a hexagonal lattice, which provides a scaffold for postsynaptic proteins (Tyagarajan & Fritschy 2014). In neurons lacking certain $GABA_A$ receptor subunits, gephyrin forms

intracellular aggregates without postsynaptic clusters, meaning that gephyrin clustering in neurons may also be regulated by other factors besides auto-oligomerization.

E domain has high-affinity binding with M3-M4 intracellular loop of the GlyR β subunit. Interaction between gephyrin and $GABA_AR$ α (1-3) and γ2 subunits are also indicated (Tretter et al 2012, Yu et al 2007). The interaction of gephyrin and $GABA_AR$ α3 subunit residue 368-376 at the intracellular loop has been thought as the key residues for the recruitment of $GABA_AR$ to postsynaptic sites (Maric et al 2014a). To modulate the excitatory transmission by gephyrin, a common binding site of GlyR and $GABA_AR$ α (1-3) with gephyrin was shown in Fig 1.1(Maric et al 2011).The residues 398-408 from GlyR β subunit have the best binding affinity with gephyrin.

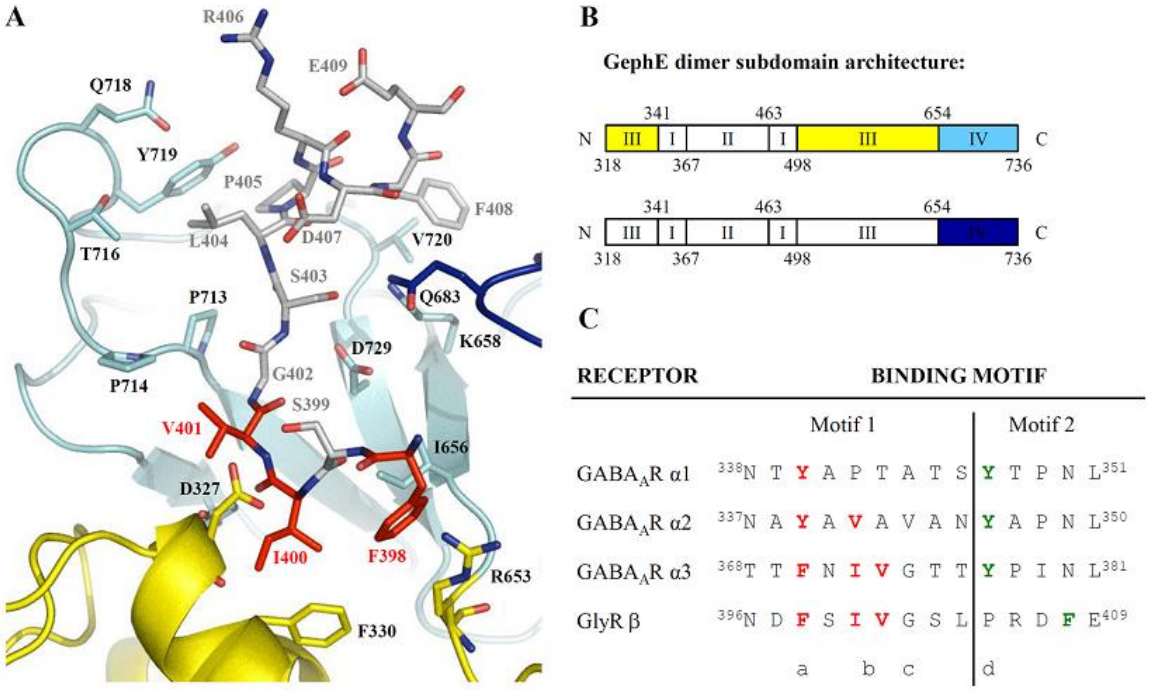

Figure 1.1 The common binding site between gephyrin and GlyR or GABA$_A$R

Reprinted from (Maric et al 2011)

A: The crystal structure of the binding interactions between gephyrin E domain and GlyR β loop was shown.

B: Subdomain of GephE dimer. **C**: Sequence alignment of binding motif recognized by gephyrin. The figure is reprinted from Maric's paper in 2011.

1.2.3 Diseases related to the imbalance between excitatory and inhibitory synapses

Since the excitatory and inhibitory transmission determines the normal function of central nervous system, there are many diseases that may be attributed to imbalance of excitation and inhibition.

Autism is a devastating neurodevelopmental disorder and it is characterized by impairments in social communication, repetitive stereotyped behaviors and language development (Sahin et al 2015). There are several different hypotheses about this puzzle. It is a disorder of multiple factors including genetic, environmental and immunological factors. One of the potential mechanisms is increased ratio of excitatory and inhibitory neurotransmission in the brain. In PSD95 knockout mice, increased repetitive behaviors, abnormal communication and social behaviors, and increased stress reactivity were observed (Feyder et al 2010). ASD mouse models also showed consistent upregulation in the dynamics of PSD95 positive spines while the clusters of gephyrin were not affected (Isshiki et al 2014). Suppression of GABA was also observed in the autism spectrum disorders. Bypassing proteasome-mediated degradation of PSD95 contributes to altered synaptic plasticity. They found multiple autism-linked genes including myocyte enhancer factor 2 (MEF2) and fragile X mental retardation protein (FMRP) mediated synapse elimination via degradation of PSD95 (Tsai et al 2012).

The mutation in gephyrin from family genetic also revealed that gephyrin has links with the neuronal development. In a family, a 357kb de novo deletion in gephrin caused limited movement, slow motor development and language delay. A 273kb de novo deletion caused

developmental delay, cyclical seizures, and behavioral issues including anxiety, obsessive compulsive disorders, tics, and impulsive behaviors (Chen et al 2014, Lionel et al 2013).

There is also a mouse model of ASD with reduced spontaneous GABAergic neurotransmission. When the mice were treated through positive allosteric modulation of GAB_AA receptors, deficits in social interaction, repetitive behavior and spatial learning improved (Han et al 2014).

Other diseases related to the imbalance of excitation and inhibition include seizures, which were reported to associate with excitotoxicity and the accumulation of glutamate (Essa et al 2013). Considering all this, maintaining balance of excitatory and inhibitory transmission is key to the normal function of brain.

1.3 Peptide mediated protein knockdown system

The manipulation of protein expression could be done at the gene level, the RNA level and also post-translational level. Endogenous protein turnover is highly regulated by clearing out misfolded, damaged and harmful protein to maintain normal cell function. The autophagy lysosome system and ubiquitin proteasome system are the most common systems that play the role to eliminating endogenous proteins at certain circumstances for the homeostasis of cells (Keller JN 2004, Laurent 2004, Macri et al 2015, Meijer 2004, Menzies et al 2011). In this thesis, the main system I used was the Chaperone-mediated autophagy (CMA).

1.3.1 Characteristics of endogenous protein degradation through autophagy lysosome system

Lysosomes are single membrane-bound organelles that are responsible for breaking down superfluous or damaged biological molecules, which are then recycled back to the cytosol as the gradients for new molecules, forming a continuous degradation-regeneration cycle (Wirawan et al 2012). Compared to proteasome, autophagy through lysosome almost has unlimited capacity. The normal function of lysosomes depends on acidic hydrolases and lysosome membrane proteins (LMPs) residing on membrane (Eskelinen et al 2003). The acidic hydrolases are responsible for breaking down large molecules into the basic units. The LMPs are responsible for acidification of lysosome lumen and internalization of substrates.

There are three types of autophagy in mammalian cells: macroautophagy, microautophagy and chaperone-mediated autophagy. Macroautophagy is the major and best-characterized autophagic pathway with specialized vacuoles (autophagosomes) for cargo transportation. This pathway initiates with engulfment of cytoplasmic components by the isolation membrane (phagophore) in the cytoplasm, which ultimately close to form autophagosomes. Then the autophagosomes mature and travel to perinuclear area to fuse with lysosomes to deliver its cargo for degradation (Bergamini et al 2004, Menzies et al 2011, Rideout et al 2004). Microautophagy is a process occurs when the lysosomal membrane invaginates to engulf components of the cytoplasm (Keller 2004).

Chaperone-mediated autophagy (CMA) so far is only identified in mammalian cells. The three major events are: (1) individual recognition of single substrates, (2) protein unfolding,

(3) translocation into lysosomes (Arias & Cuervo 2011, Bejarano & Cuervo 2010). The recognition step is unique because the proteins are recognized one-by-one by a cytosolic chaperone called heat shock cognate protein of 70kDa (hsc70) through a recognition motif. Only proteins with a pentapeptide KFERQ or KFERQ-like motif will be recognized (Dice 1990). This motif later is recognized as the CMA targeting motif (CTM). Removal of KFERQ from ribonuclease A abolished the lysosomal degradation responding to nutrient deprivation (Dice et al 1986). And insertion of this sequence into proteins that are not degraded by lysosome is sufficient to induce their degradation through CMA (Koga et al 2011). CTM is based on the physical properties of the amino acids rather than specific amino acids. All CMA-targeting motifs consist of a positively charged residue (K, R), a negatively charged residue (D, E), a hydrophobic residue (I, L, V, F), another positively charged or hydrophobic residue, and a critically important Q on either side of the pentapeptide. The location of the CMA targeting motif is quite flexible, and it can be located in the C terminal, N terminal or in central regions of the protein as long as it can be exposed to the chaperone protein. Since the CTM is always there, it is regulated through partial unfolding of the proteins and dissociation of interacting proteins masking the motif and release from sub compartments to be exposed to chaperone. For the number of CTM, one motif is sufficient for lysosomal targeting though some proteins contain more than CTM. Those with different CTM may be available for recognition in different conditions (Bejarano & Cuervo 2010, Kaushik & Cuervo 2012, Liu et al 2015, Massey et al 2004).

The chaperone protein hsc70 is a multifunctional protein that participates in many other cellular functions such as protein folding, protein complex disassembly. So far hsc70 is the only

chaperone identified for CMA targeting (Chiang et al 1989). Besides targeting for CMA, hsc70 also targets proteins with the KFERQ-like pentapeptide to late endosomes for endosomal microautophagy (e-MI). It is also indicated to mediate protein degradation by macroautophagy via chaperone assisted selective autophagy. The co-chaperones such as CHIP, BAG1, and hsp90 may modulate the targeting to a specific pathway when associated with hsc70. The complex then delivers the substrates to lysosomes and may assist in unfolding of the proteins which is unnecessary for binding. But unfolding is definitely required for translocation into lysosomal lumen. The substrate protein will bind to the cytosolic tail of the single-span membrane protein lysosome-associated membrane protein type 2A (LAMP-2A) (Cuervo & Dice 1996). And this binding is the limiting step for CMA. LAMP-2A has different forms, and substrate will bind to monomeric LAMP-2A to assemble into a high molecular weight complex. The multimerization is necessary for translocation. Once the substrates are released into the lysosomal lumen from the complex, LAMP-2A will dissociate into monomers again, forming an active multimerization and disassembly. Then the substrates are degraded by the hydrolyzed in the lumen into building blocks (Bejarano & Cuervo 2010). Though the three kinds of autophagy share different characteristics, they also have some cross-talks to have the compensatory mechanisms.

1.3.2 TAT as a delivery tool

The conventional categories of drugs are divided into two groups: the small molecule drugs and larger biological molecules including peptides and proteins. The challenge for small molecule drugs is mainly the reduced selectivity due to their sizes which may result in the side effects by off target actions. For larger proteins as therapeutics, they could be specific due to many interactions with the targets, but the shortness lies on low bioavailability, metabolic instability

and poor permeability (Otvos et al 2014). Peptides that have less than 50 amino acids have been emerged as a new trend for drug discovery for decades trying to combine the small molecules and large proteins. In addition, more than 7000 natural peptides have been identified and they play multiple roles in physiology including neurotransmitter, hormones, growth factors, ion channel ligands (Giordano et al 2014). More than 140 peptides are being evaluated in clinical trials. But the peptide as a therapeutic approach has its pros and cons. The strength include high selectivity and potency, good efficacy, tolerability and standard synthetic protocols while the weakness include chemically and physically instable, fast elimination, low membrane permeability and so on.

To conquer the problem of the low membrane permeability, a group of cell penetrating peptide (CPP) such as TAT, penetratin, Arg-9 and pep-1 has been conjugated to improve delivery. Here I will focus on TAT considering its wide use in our lab. TAT (trans-activating transcriptional activator) is from human immunodeficiency virus 1 (HIV-1) and is the first CPP discovered in the lab in 1988 when it was found to be taken up by several cell types in culture (Frankel & Pabo 1988). And later the residues from 48-60 were found to have the penetrating capability (Ebrahimi-Fakhari et al 2014). Any deletion within 49-57 showed reduced membrane translocating activity (Park et al., 2002). Thus the residues from 48-57 are widely used as the CPP. In this thesis, TAT will be referred as residues 48-57 unless it is otherwise stated.

How CPP penetrates into the cells remains unclear but there are several different hypotheses for the penetration due to their classification. The first class is cationic with arginine-rich sequence consisting of at least eight arginine residues (Tunnemann et al 2008). The positively charged

arginines develop strong electrostatic interaction with negatively charged glycoproteins on the membrane, and the disturbance forms a transient pore on the membrane for the peptide to directly translocate into the cells. Other direct translocations include carpet-like model, inverted micelle formation (Figure 1.2). Another proposition is that the electrostatic interaction results in endocytosis of the peptide through a receptor independent way (Derossi et al 1996). And the endocytosis could be divided into different subtypes: phagocytosis, clathrin-mediated endocytosis, caveolin-mediated endocytosis, micropinocytosis and clathrin-caveolin-independent endocytosis (Figure 1.2).

The application of TAT as a delivery tool has been used in many different cargos such as DNA, proteins, and small molecules both in vitro and in vivo to obtain better membrane permeability. It is also systematically used to delivery cargos to the brain by better crossing the blood brain barrier. TAT was applied in DNA plasmid transfection (Rudolph et al 2003). Fusion of TAT to shRNA and siRNA also showed more stable and efficient gene silencing compared to lipofectamine without obvious cytotoxicity (Arthanari et al 2010). Besides DNA and siRNA, TAT has been used to transport antibodies into the cells. For instance, tumoricidal immunoglobulins conjugated with TAT was found to have a better cellular uptake (Anderson et al 1993). Other proteins that have been conjugated to TAT include protein-based vaccines, stimulating T cell responses (Kim et al., 1997).

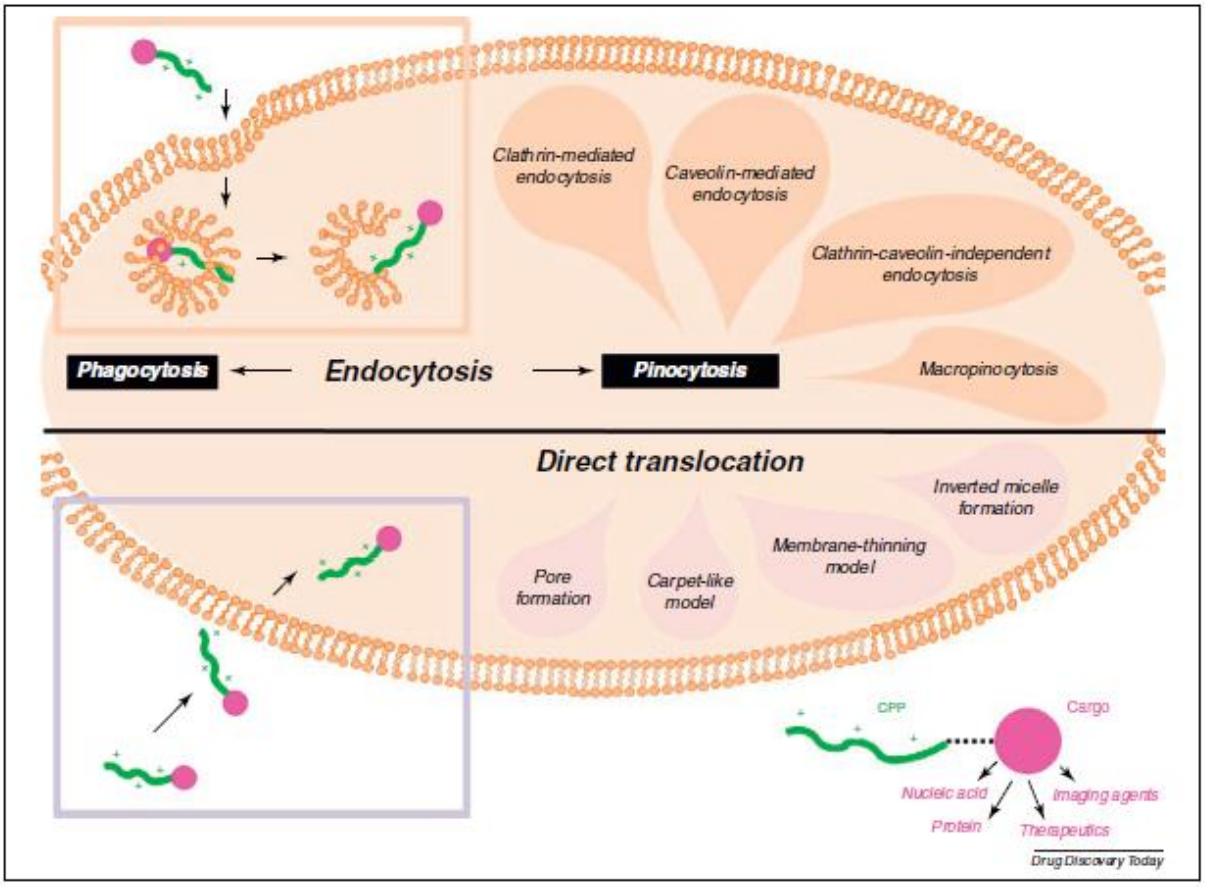

Figure 1.2 Differerent mechanisms for the internalization of cell penetrating peptide (CPP)

Printed from (Rizzuti et al 2015)

CPP can cross the membrane by the direct translocation pathway which is energy independent based on the peptide and membrane interaction. CPPs can also penetrate using different endocytotic pathways.

1.3.3 Issues about peptide as a drug

The main concerns in development of peptides were toxicity, immunogenicity and stability. There are already methods to improve the peptides. For examples, Incorporation of D-amino acids made the peptides protease resistant. Other chemical modification such as changing the peptide backbones and cyclization would also enhance the stability (Gentilucci et al., 2010).

Several groups have designed models to analyze the toxicity of the peptides. The Raghava group (Gupta et al 2013) developed an in silico method to predict the toxicity of peptides having 35 or fewer residues. They analyzed 1805 known toxic peptides and 3993 non-toxic peptides collected from various databases and found out that composition of Cys, Pro, Asp and His was found to be higher in those toxic peptides (Gupta et al 2013). The platform they built is ToxinPred which allows users to predict toxicity for the submitted peptides and their mutants. Whether the suggested mutant had lower toxicity without comprising the efficacy had to be tested.

Many studies both in vitro and in vivo also assessed the toxicity of CPPs. Among different groups of CPPs, the toxicity was found to decrease from oligoarginine to penetratin to TAT (El-Andaloussi et al., 2007). But with cargoes, that could be a completely different story. TAT fused to several peptides that could block the main pro-apoptotic pathway was found to be toxic when the concentration was over 10uM while TAT alone up to 100uM was harmless to the cells (Cardozo et al., 2007).

1.4 Specific aims

Our lab has developed a novel technique for rapid and reversible protein knockdown through a small peptide. The peptide consists of three parts. The first is a cell penetrating peptide sequence. The second is the binding sequence to bind specifically to the targeted protein. The third is either a CMA or degron targeting motif that can drag the peptide-targeted protein complex to lysosome or proteasome for degradation. This small peptide-mediated knockdown is rapid and not limited to spatiotemporal control comparing to other manipulations at DNA and RNA level. This method also extends the convenience and possibilities of clinical application compared to other post-translational modification that require genetic engineering or viral transfection (Shelly et al 2014).

Based on these advantages of the technology and the importance of PSD95 and gephyrin in the respective maturation and maintenance of excitatory and inhibitory synaptic structure and functions, I reasoned that it would be useful if we can develop and optimize the peptides that can rapidly and reversibly knockdown PSD95 or gephyrin. I further hypothesize that through controlling the expression levels of these scaffold proteins in neurons by treating these neurons with these peptides in vitro and/or in vivo, we may be able to regulate the levels of synaptic excitation and/or inhibition, thereby achieving our primary goal of fine tuning the balance of synaptic excitation and inhibition. To reach this goal, I have attempted to realize the following aims:

Aim 1: Characterizing the efficacy of peptide that knocks down PSD95.

Aim2: Design and characterizing the peptide that knocks down gephyrin.

Aim3: Develop strategies to increase the efficacy and reduce the cytotoxicity of peptides.

Chapter 2: Methods and materials

2.1 Primary neuron culture, treatment and collection

2.1.1 Primary neuron culture

Primary neurons were prepared from embryos of Sprague-Dawley rats when the mom was pregnant for 18-19 days. The mom was sacrificed and the pubs were removed from the uterus and placed in the sterile 10cm dish (Corning, 0877222). Then the brain was carefully removed and cortex was dissected and put into new 10cm dish filled with ice-cold HBSS (Invitrogen, 14170-112). The cortex was digested with pre warmed 0.25% Trypsin-EDTA (Invitrogen, 25200-072) at 37℃ for 15minutes. Trypsin was inactivated by washing three times with DMEM (Sigma, D6429) supplemented with 10% FBS (Invitrogen, 12483020). Then the cells were centrifuged and re-suspended in neuro basal media (Invitrogen, 21103-049) containing 2% B-27 supplement (Invitrogen, 17504-044) and 0.5mM GlutaMax ((Invitrogen, 35050-061) by different sizes of pipettes. Then cell number was counted and they were diluted in certain neuro basal media before plating on Poly-D-lysine (PDL) coated plates. The cells were cultured in the incubator at 37℃ with 95% O_2 and 5% CO_2 and two thirds of the media were replaced every 3-4 days to guarantee the nutrition until it was ready for use.

2.1.2 Treatment of primary cultured neurons

Generally, the neurons were used at 14-16 day for in vitro experiments. Before treatments, the media in each well was averaged to 1mL if it was 12-well plate and the redundant media was collected as the conditioned media. The stock solution of peptides was added directly to the media at the desired concentration and after desired time of treatment, the media was sucked out by vacuum and 1mL of conditioned media was added back until the cells were ready to be collected.

2.1.3 Collection of neuron cultures

To collect cultured neurons for immunoblot analysis, the media was sucked out and washed with 1X ice-cold PBS (phosphate-buffered-saline) and 300uL of ice-cold RIPA (radio-immuno-precipitation-assay) lysis buffer was added into each well of 12-well plate. The protease and phosphatase inhibitors (Thermo Fisher Scientific, 78442) were added into RIPA buffer right before use. Then the whole plate was shaken in the cold room (4°C) for about 30min until all the neurons fell off the plate and the neurons were collected into 1.5mL Eppendorf tube. And the tube was centrifuged at 13,000rpm for 10min at 4°C to precipitate cell debris and the supernatant as the protein sample was collected into new 1.5mL Eppendorf tube for concentration determination. If the immunoblot was not done immediately following the concentration determination, the supernatant was stored in -80°C for later use.

2.2 Peptide synthesis

2.2.1 AutoSpot synthesis of peptide arrays

Filter cellulose paper acquired from whatman ® 50 was used to synthesize high number of peptide arrays by the peptide synthesis group in our lab. First Fmoc (Fluorenylmethyloxycarbonyl)-β-Alanine was used to amine-functionize the filter paper and then the peptides were synthesized from C-terminal to N-terminal, with C-terminal anchored to the membrane. Fresh amino acid solutions were made for everyday use. Addition of each amino acid can be divided into coupling reaction, Fmoc-removal, membrane staining. The dried membrane was put on an inert surface and the AutoSpot program would deliver the required volume of each amino acid solution to all the positions. Then the Fmoc protection group was removed by 4 short wash with DMF (dimethylformamide) followed by two other 5 minutes wash with 20%

piperidine in DMF, three more quick washed with DMF and two more washes with ethanol. Then the membrane was stained with bromophenol/ethanol staining solution until the membrane showed a homogenous light blue color to make sure the free amino acid group was added on successfully. Then the membrane was gone through two washes with ethanol and was left to be dried in the airstream. The peptide chain was built up by repeating the coupling cycle. Once the final amino acids were added, the peptides side-chain protection groups was removed by TFA cleavage cocktail solutions with 5% deionized H2O, 3% Triisopropylsilane, 1% Phenol, 1% DCM and 90% TFA. Finally the membrane was washed 5 times by DCM for 1min each and then washed 5 times by ethanol for 1min each. Dry the membrane in the airstream. Each spot on the finished membrane had 11amino acids and contained all the mutated forms of glyR β 398-408 as proposed.

2.2.2 Peptide synthesis in powder form

Besides the peptide arrays, other peptides were also synthesized in our lab using Prelude peptide synthesizer (Protein Technologies Inc.). The principle was similar to the synthesis of peptide arrays, that the peptide was synthesized from C terminal to N terminal. But this was a solid-phase peptide synthesis (SPSS) and polyamide resins were used as the solid support that amino acids could be attached to. Briefly, the SPSS contained repeated cycles of deprotection-wash-coupling-wash and a single N protected unit was coupled to the free N terminal amine of the solid phase attached peptide from last cycle. After the final cycle, the protective groups were also removed from the side chains of the peptides and the peptides were cleavage by TFA from the solid support. Then the peptides were purified by HPLC. Dry peptide powder was stored in -80℃ and stocked solutions were obtained by dissolving the peptides in sterile ddH_2O at the

concentration of 25mM or 5uM. And they were aliquoted to avoid freeze-thaw cycles. The sequence of peptides used in the thesis was listed in Table 2.1.

2.3 Protein analysis

2.3.1 Protein Concentration determination

The concentration of supernatants as described in Chapter 2.1.3 was determined by Lowry method using protein assay kit (Bio-Rad, 500-0112). Briefly, protein standards were made from powder BSA and the stock solution of BSA was 10mg/mL, dissolved in RIPA buffer. Then it was serially diluted to the concentrations of 5mg/mL, 2.5mg/mL, 1.25mg/mL, 0.625mg/mL, 0.312mg/mL and there was RIPA buffer alone as blank control. Reagent A and S were mixed first at the ratio of 1mL to 20uL. Then 100ul mixture of reagent A and S was added to the plastic cuvette followed by 10uL of each protein standard or protein sample and then 890uL of reagent B was added to the cuvette. The mixture was rested in dark at room temperature for about 15 minutes for the color to develop then the absorbance was measured at wavelength of 750nm by Biochrom Ultraspec 60 (Fisher Scientific, BC80710008). The standards were measured first to make a standard curve and the samples were measured and the concentration was calculated based on the standard curve.

2.3.2 Electrophoresis and western blot analysis

Same amount of protein samples were mixed with 6X sample buffer, boiled at 95℃ for 5min and then centrifuged at 14,000rpm for 1min at room temperature. The proteins were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using electrophoresis system (Bio-Rad, 1658000). The top of the SDS-PAGE gels were 5% stacking gels (2.82mL of

ddH_2O, 0.83mL of 30% acrylamide solution, 1.25mL of 0.5M Tris (pH 6.8), 50uL of 10% SDS, 50uL of 10% APS and 5uL of TEMED for each one) and the bottom were 10% acrylamide separating gel (4.0mL of ddH2O, 3.3mL of 30% acrylamide solution, 2.5mL of 1.5M Tris (pH 8.8), 100uL of 10% SDS, 50uL of 10% APS and 5uL of TEMED for each one). 10-well or 15-well comb was inserted into the stacking gel to make loading wells.

Proteins were transferred to 0.45um polyvinyl difluoride (PVDF) membranes (Bio-Rad, 162-0177) after electrophoresis. And the membrane was blocked with 5% BSA in TBST (Tris-buffered saline with Tween-20, containing 50mM Tris-HCl, 150mM NaCl, and 0.1% Tween-20) for 1h at room temperature and then incubated with primary antibody overnight at $4^{\circ}C$. The membrane was washed 3 times of 5 min each with TBST before incubating with HRP (horseradish peroxidase) conjugated secondary antibody for 1h at room temperature. After another three washes of TBST, the membrane was subject to 1mL Luminata Crescendo Western HRP substrate (Fischer, WBLUR0500) and the bands were detected in the imager (Bio-Rad ChemiDoc MP System, 1708280).

The primary and secondary antibodies used in the thesis were listed in Table 2.2.

2.3.3 Purification of GST fused recombinant protein

The GST-GephE (GST-Gephyrin E domain) fused protein was purified with Glutathione Sepharose 4B (GE Healthcare Life Sciences, 17075605) in a gravity flow column following the manufacturer's instructions.

Briefly, the sequence of GephE (Gephyrin E domain) was amplified via PCR and the enzymes chosen were BamHI and EcoRI. The sequences of primers were as following.
Primer for GephE sense: 5'-CGC GGA TCC ATG TCT CCT TTT CCT CTG- 3'
Primer for GephE antisense: 5' –CCG GAA TTC TCA TAG CCG TCC GAT GAC -3'
Then the sequence of GephE was inserted into vector pGEX4T-1 and the right clone was confirmed by sequencing.

The reconstructed plasmid containing GephE was transformed into BL21 bacteria and cultured in 300mL LB media at 37℃ in a shaker at 250rpm until the absorbance at 600nm of the culture reached 0.6. And the IPTG (isopropyl β-D-thiogalactoside) (BioShop, IPT001) was added to a final concentration of 100uM to induce the synthesis of proteins for 5h at 37℃ in the shaker. The culture media then was centrifuged at 4,000rpm for 20min at 4℃ and the pellet was saved.

To purify GST fused proteins, bacteria pellets were lysed in 15mL ice-cold PBS containing protease and phosphatase inhibitor (Thermo Fisher Scientific, 78442) using sonication. Then the protein samples were centrifuged at 13,000rpm at 4℃ for 10min and the soluble part was transferred to a new tube to incubate with Glutathione Sepharose 4B equilibrated with 20 mM Tris pH 7.5, containing 0.1 % Triton X-100for 1h at room temperature. Then the mixture was loaded in the column and drained by gravity. The flow through was collected for analysis. The beads were washed three times with PBS and the flow through were also collected into new tubes. Then the GST purified protein was eluted by 6mL elution buffer with 10 mM reduced glutathione in 50mM Tris pH 8.0. The flow through was collected into 1.5mL Eppendorf tube as the recombinant protein.

The flow through of recombinant protein was separated on a SDS-PAGE gel as described in Chapter 2.3.2. After running, the separating gel was stained in Coomassie Blue solution for 2h at room temperature in a shaker and then distained in the distaining solution overnight to see if there is band on the desired size. Following Coomassie Blue staining, the samples were also separated on SDS-PAGE gels and transferred to PVDF membrane to be recognized for GST and gephyrin antibodies. The purified protein was store at -80℃.

2.3.4 Peptide-Protein interaction assay

This assay was for the screening of peptides for the recombinant protein. The membrane with high number of peptides was activated by washing with ethanol for 10min. Then the membrane was washed 3X 5min with TBST and blocked with 5% BSA for four hours at room temperature. Membrane then was washed once with TBST for 5min and incubated with recombinant protein at a desired concentration overnight at 4°C. The membrane was wash 3 times with TBST for 20min each. Then the membrane was incubated with primary antibody and secondary antibody and visualized as described in Chapter 2.3. 3.

2.4 Cell death assay

Cell toxicity was detected by the release of LDH (lactate dehydrogenase) released from the cytosol to the supernatant using Roche Toxicology Assay kit (Sigma, 04744934001) following the manufacturer's instructions. The catalyst was in powder form and was dissolved in 25mL ddH_2O and stored at -20°C after aliquot. The dye was aliquoted and stored at -20°C, too. The

reaction mixture contained 22uL of catalyst when the dye was 1mL. Then equal amount of substrate solution was mixed together with the reaction mixture.

50uL of conditioned media was collected into a Costar 96-well plate (Fisher, 0720035), 50uL of LDH assay reaction mixture was added to the media. 50uL of ddH_2O was used as negative control. The plate was wrapped with aluminum foil and shaken mildly on the shaker (300rpm, 25°C) for 20min. Then the background absorbance was measured at 690nm and the total absorbance was measured spectrophotometrically with a plate reader. And total absorbance deducted by background absorbance was used to quantity the release of LDH.

2.5 Data analysis

Western images were analyzed by the Image Lab and when the band was circled, the software recognized the background and the band. Their intensity was shown for comparison and analysis.

Values are expressed as mean ± SEM. One-way ANOVA was used for statistical analysis and P values less than 0.05 was considered statistically significant.

Table 2.1 Synthetic peptide sequence

Synthetic peptide	Target protein	Sequence
TAT	N/A	YGRKKRRQRRR
TAT-NR2B9C-CMA	PSD95	YGRKKRRQRRR-KLSSIESDV-KFERQKILDQRFFE
TAT-NR2B9C (NA1)	PSD95	YGRKKRRQRRR-KLSSIESDV-
TAT-NR2B9C-QREFK	PSD95	YGRKKRRQRRR-KLSSIESDV- QREFK
KFERQ-TAT-NR2B9C	PSD95	KFERQ- YGRKKRRQRRR-KLSSIESDV
TAT-NR2B9C-PEG12-CMA	PSD95	YGRKKRRQRRR-KLSSIESDV-PEG12-KFERQKILDQRFFE
TAT-Geph1-CMA	Gephyrin	YGRKKRRQRRR-FSIVGSLPRDF-KFERQKILDQRFFE
TAT-Geph1-QREFK	Gephyrin	YGRKKRRQRRR-FSIVGSLPRDF- QREFK
TAT-Geph1-PEG12-QREFK	Gephyrin	YGRKKRRQRRR-FSIVGSLPRDF -PEG12-QREFK
TAT-NR2B	DAPK1	YGRKKRRQRRR- KKNRNKLRRQHSY
TAT-SRC-CMA	N/A	YGRKKRRQRRR-KLSSIEADA-KFERQKILDQRFFE

Table 2.2 Primary and secondary antibodies

Primary antibody	Company	Catalogue#	Dilution
PSD95	Thermofisher	MA1-046	1:5000
Gephyrin	Synaptic Systems	147 111(SY)	1:5000
β-actin	Abcam	AB8227	1:3000
Secondary antibody	**Company**	**Catalogue#**	**Dilution**
Anti-Mouse IgG (HRP conjugated)	Perkin-Elmer	NEF822001EA	1:5000
Anti-Rabbit IgG (Goat), HRP-Labeled	Perkin-Elmer	NEF812001EA	1:5000

Chapter 3: Results

3.1 Peptide design and efficacy test

3.1.1 In vitro test of peptide that targets PSD95

PSD95 is the scaffolding protein in excitatory synapses and is relatively abundant in the PSD. PDZ domain of PSD95 was found to bind with nine amino acids of NR2B c-terminal. Our lab has already designed the peptide that targets PSD95 based on its binding site in the NR2B c-terminal (Shelly et al 2014). The peptide is named as TAT-NR2B9C-CMA and the sequence is YGRKKRRQRRR-KLSSIESDV-KFERQKILDQRFFE. However, its efficacy has not been properly characterized yet. The first experiment I did was to test the efficacy of the peptide.

The knockdown effect was checked following bath application to cultured cortical neurons for 4h, 8h, 12h, and 24h at concentrations of 3.125uM, 6.25uM, 12.5uM and 25uM. I found out that PSD95 could be knocked down dramatically after being treated with TAT-NR2B9C-CMA for 8h and 24h at the concentrations of 12.5uM and 25uM (Fig3.1). Even at 4h, PSD95 was almost knocked down to 48% ± 12.5% of the untreated control levels (Fig 3.1 A and B), revealing a rapid, and efficacious knockdown as expected. The knockdown effect was dose dependent in general.

However, when checking the morphology of the cultured neurons under microscope before collecting the samples for western, I found that most of the neurons looked unhealthy. I suspected that these peptides may have some cytotoxic effects. I therefore used LDH assay to further analyze this possibility. LDH assay was applied to detect how much lactate dehydrogenase was released from the cytosol of damaged cells, thus reflecting the degree of

cytotoxicity. TAT-NR2B9C-CMA showed toxicity if the treatment was longer than 12h at 6.25uM, and toxicity was shown if the treatment was longer than 8h at 12.5uM. If the concentration was 25uM, toxicity could be seen even at 4h (Fig 3.2A). I observed that the longer time and/or higher concentrations of the treatment, the higher degree of cytotoxicity. Thus, the cytotoxicity was positively correlated to time and concentration. It was also interesting to note that at 4h, 8h, and 12h, the cytotoxicity at 12.5uM was consistently lower than that at 25uM. But at 24h, the level of cytotoxicity was almost the same at 12.5uM and 25uM. One possible reason could be that at these points, the damage to the cells reached its maximum. Here TAT-NR2B9C was used as a control which was not supposed to knock down PSD95 and at the same time and concentration range, TAT-NR2B9C showed no toxicity to the cells (Fig 3.2B). Thus, whether the toxicity of the TAT-NR2B9C-CMA was a result of the intrinsic property of the peptide or a consequence of PSD95 knockdown remained unclear at this moment.

3.1.2 Search for gephyrin binding peptide

To develop a peptide that specifically knock down gephyrin in inhibitory synapses, I first designed a small peptide that would bind with gephyrin. The Maric group found that GlyR β subunits bind with gephyrin with good binding affinity (Maric et al 2011). The structure of binding between GlyR β subunits and gephyrin has also been discovered as described in Chapter 1.2.2. Amino acids from 398-408 of GlyR β subunit were thought to be the residues that played a critical role in binding with gephyrin. By looking at the interaction interface in the structure, we hypothesized that S(399), I(400), V(401), G(402), L(404), P(405), F(408) were the critical amino acids for the binding. I assumed that if those amino acids were switched to some other residues, I might get better binding affinity due to the size and charger characteristics of the amino acids. To

test this idea, I mutated S(399) to either K or L, I(400) to F, V(401) to I, G(402) to A, L(404) to F, P(405) to either K or V, and F(408) to either W or I. Based on the different mutation sites, I generated all potential 432(=3*2*2*2*2*3*3) mutants (including the original one) shown in Table3.1.

3.1.3 Screening of peptides for gephyrin using AutoSpot synthesis of peptide arrays

After designing the 432 mutant peptides, I did the first round of screening using AutoSpot synthesis of high density peptide arrays. All the mutant peptides were synthesized onto the membrane and each dot represented one peptide as described in Chapter 2.2.1. Then I used the purified recombinant gephyrinE domain (0.1ug/mL) to overlay with those peptides on the membrane (Fig 3.3 A and B) and found that there were 40 peptides (Table 3. 2) showing various degree of binding. For the first round of screening, the concentration of GephE domain was quite high and there might be some false positive results. Second round of screening was then done at various doses of recombinant GephE: 0.1ug/mL, 0.05ug/mL, 0.001ug/mL (Fig 3.3C). And 5 peptides (FSIVGSLPRDF, FSIVGSLPRDI, FSIVGSFPRDF, FSIIGSLPRDF, and FSIIGSLPRDI) that showed consistent binding were shown in Table 3.3. In this experiment, recombinant GST was used to overlay with the peptides as a negative control and another negative control was in absence of recombinant protein. Both negative controls showed no binding with the peptides (Fig 3.3D).

3.1.4 In vitro test of peptide that targets gephyrin

While searching for the best candidate that binds with gephyrin, I synthesized the peptide (TAT-Geph1-CMA) with the original binding sequence to do the efficacy test. Similarly as the peptide for PSD95, the knockdown effect was checked following bath application of the peptide to cultured cortical neurons for 4h, 8h, 12h and 24h at concentrations of 3.125uM, 6.25uM, 12.5uM and 25uM. The knockdown effect was also dose dependent. At 4h, the knockdown was obvious at concentration higher than 6.25uM. At 25uM, only 21 ± 0.4% of gephyrin was left. At 12.5uM, 37 ±5.3% was left. At 6.25uM and 3.125uM, the number became 62.9 ±13.3% and 78.1 ±18.1% (Fig 3.4B). When the treatment time reached 8h, 12h and 24h, the dose dependent manner was still there and the knockdown effect was comparable to the corresponding concentration at 4h (Fig 3.4).

When I checked the morphology of the cultured neurons, most of the neurons looked unhealthy. Then LDH assay was applied to determine the degree of cytotoxicity. And the result (Fig 3.5) showed that TAT-Geph1-CMA was even more toxic than TAT-NR2B9C-CMA. After 4h, the peptide started to show toxicity when the concentration was higher than 6.25uM. At 8h and 12h, the LDH levels increased more and at 24h, the LDH level was similar between 6.25uM, 12.5uM and 25uM and the LDH went up even at 3.125uM. Similar to TAT-NR2B9C-CMA, the equal amount of LDH at 6.25uM, 12.5uM and 25uM might be due to the saturation of the toxicity.

3.2 Identification and reduction of peptide toxicity

3.2.1 Identification of peptide toxicity

As shown in chapter 3.1, I found both peptides that targeted PSD95 and gephyrin showed toxicity in cultured neurons. Since I was not sure whether it was a common issue for all knock down peptides or specific to these two individual peptides, I collected some other peptides targeting different proteins that had been synthesized in our lab to test their toxicity. TAT-NR2B-CMA was designed to target DAPK1 (Death Associated Protein Kinase1) while TAT-NR2B was used as its control peptide (Shelly et al 2014, Weihong et al 2010). TAT-β syn-degron was designed to target α-synuclein which accumulates in Parkinson's diseases. TAT-β syn91-degron and TAT-β syn were used as the control peptides. TAT-NR2B9C-CMA was to target PSD95 as described in Chapter 3.1.1. TAT-src-CMA was its control peptide. The result was shown in Figure 3.6. The toxicity of the peptides was measured from the conditioned media treated with the peptide at 10uM, 20uM or 30uM for 24h. The toxicity of peptides seemed to be some common issues when the concentration went up to 30uM or even 20uM. TAT alone had no toxicity to the neurons even at the concentration of 30uM. I also tested several other peptides including TAT-mHTT-CMA and TAT-R2-degron which also showed similar trend (Data not shown here).

By comparing those peptides, I was also aiming to find out the cause of toxicity. TAT alone had no toxicity up to 30uM. Actually, TAT alone was found to be neuroprotective in both neuronal cultures against oxygen-glucose deprivation (OGD) and reducing infarct volume in a permanent middle cerebral artery occlusion (MCAO) rat stroke model (Milani et al 2016, Krautwald et al 2016). But the mechanism how it protected the damage remained unclear. After excluding the

possibility of TAT, I looked into the binding sequence. TAT-NR2B showed toxicity at 30uM and TAT-β syn already showed toxicity at 20uM, while TAT-NR2B9C was of little toxic effect to the neurons even at 30uM. Another fact was that TAT-NR2B9C was also reported by several groups to have protection to the neurons from NMDA induced excitotoxicity and ischemia (Aarts et al 2002). TAT-NR2B was composed of 24 amino acids and TAT-NR2B9C had 20 amino acids and TAT-β syn consisted of 20 amino acids. The main difference of the three peptides were reflected on the composition of the amino acids namely the primary sequence. Raghava group has found out that composition of Cys, Pro, Asp and His was higher in toxic peptides (Gupta et al 2013). Thus the peptide binding sequence appeared largely responsible for the toxicity of the peptides.

Then the CTM was investigated and until now, all the peptides with CMA containing fused 3 targeting motifs that had 14 amino acids showed toxicity when the concentration went up to 20uM. For the degron as the CTM (which only has 4 amino acid residues), TAT-β syn91-degron had no toxicity. In contrast, TAT-β syn started to show toxicity even at 10uM. I speculated that those peptides with CMA might be due to the length of the peptides since the length would top up to more than 35 amino acids or too much CMA would compromise the function of lysosomes and led to the damage of cells. Comparing TAT-β syn, TAT-β syn-degron and TAT-β syn91-degron, TAT-β syn-degron was more toxic than TAT-β syn whereas TAT-β syn91-degron showed no toxicity. So the contribution of degron to the problem was hard to say.

Considering the pattern of the toxicity, I thought that the length of CTM might have contributed to the cytotoxicity to some degree. The longer of the length, there were more chances to be toxic. But there were also exceptions to it. Also three combined motifs of CTM might be another issue.

3.2.2 High dose, short time treatment

Besides the intrinsic properties of the peptides such as length and sequence, I also noticed that the peptides showed toxicity only when they reached certain concentrations and certain treatment periods. Maybe the concentration was too high and the time was too long that compromised the integrity of the plasma membrane and/or the function of lysosomes and proteasomes. As was reported, peptide was degraded into smaller fragments (Aarts et al 2002) and the fragments might also be potentially toxic. One way to minimize these was to modify the intrinsic properties of the peptides so as to increase their binding affinity, and thus less peptide was used to get the same knockdown effect. Alternatively, reduced toxicity may also be achieved by optimizing the protocols of peptide treatment without altering the sequences of the peptides.

As described in Chapter 3.2.1 and 3.2.4, the protocol I used was to treat the neurons with peptide for 24h and then collected them at different concentrations. To reduce the treatment time at high concentrations and avoid the interferences of degraded fragments, the first thing I tried was to use a high dose with shorter period of treatment. Taking TAT-NR2B9C-CMA as an example, 25uM or 10uM was used as high concentrations. The stock solution of peptides was diluted in the cultured media to get the desired concentration of 25uM or 10uM which was marked as the high concentration. After two hours of treatment, the media was sucked out and changed back to the peptide-free conditioned media. The cells were collected for immunoblot analysis and the

media was collected for LDH assay after waiting for another 4h, 6h or 8h. The results were shown in Figure 3.7. At 25uM, if the cells were treated for 2h with peptide and then waited for 6h or 8h, the PSD95 protein was almost gone, too. But if the waiting time was only 4h, the knockdown effect was minor. At the same time, LDH level already went up after waiting for 6 or 8h correspondingly. At 10uM the LDH level was comparable to the control while there was about 50% knockdown. Thus, I found that in the high dose and short time treatment protocol, while the concentration at 25uM remains seemingly toxic, peptide given at 10uM seems possible to keep the knockdown effect without obvious toxicity.

3.2.3 Low dose, multiple treatments

We next tried the low dose with multiple treatments. The idea was that the neurons were exposed to the peptides at the concentration of 5uM and then the media was aspirated in every two hours and changed back to conditioned media followed by adding a new batch of peptide. This was repeated by 5 times and cells and media were collected after, which was referred as the wash protocol. I also tried another treatment regimen, referred as the no wash protocol, in which the procedure kept the same as the wash protocol, but the media was not changed. It meant that the peptides were added every two hours and peptides would accumulate to 25uM finally if they were not degraded nor absorbed. The cells and media were collected for analysis two hours after the last addition of peptide. The results were shown in Figure 3.8, and revealed that in the no wash protocol, PSD95 was almost knocked down completely but the corresponding LDH was also high. For the no wash protocol, about 74±13% was still left, and the LDH level stayed normal, meaning that the elimination of LDH also compromised the knockdown effect. Thus, the low dose for 5 times treatments would not solve the toxicity problem.

3.2.4 Modified purification procedure

Even though the high dose short time treatment protocol and low dose, multiple treatments could reduce the toxicity, but the knockdown effect was also compromised to certain degree. To look for additional reason that causes peptide toxicity, I next turned to the contribution of peptide purity.

As described in Chapter 2, a lot of reagents were involved in the synthesis of peptides such as HoBt and TFA which could potentially toxic and hazardous. The purity of the peptide after the standard protocol could reach 95%. In order to reach a higher purity, I repeated the purification process. The purity could reach more than 99% after secondary purification. LDH test of TAT-NR2B9C peptide after re-purification showed the same toxicity as the one with purity of 95% (Figure 3.9), which meant that the purity of the peptide at 95% didn't significantly contribute to the toxicity.

As was in the standard protocol, the powder was in a form of TFA salt. To get rid of the influence of the TFA, TFA was extracted through a process referred to as desalination. After the desalination process, the texture of the peptide powder changed to more fluffy form and easier to be dissolved in ddH_2O. The toxicity was tested and the LDH level was the same as the original TAT-NR2B9C-CMA before desalination (Figure 3.9). Similar with high purity, desalination did not help to reduce the toxicity.

Higher purity and desalination didn't contribute to the reduction of toxicity, reflecting that the quality of peptides obtained with the standard protocol was good enough. The toxicity was not due to the chemicals contained in the peptide. I then turned my efforts on the potential contribution of the intrinsic properties of the peptides through changing the structures of the peptides as discussed below.

3.2.5 Addition of linker

The peptide we constructed contained three parts: cell penetrating peptide sequence, binding sequence and CMA targeting motif. The three parts are supposed to work independently to play their roles. The concern was that those parts might interfere with each other leading to the unexpected property of the peptides. Addition of linker may space each part and generate more flexibility.

PEGylation modifies protein, peptide or non-peptide molecule by linking one or more polyethylene glycol (PEG) chains (Veronese & Pasut 2005). PEG is a linear or branched polyether terminated with hydroxyl group and the general structure was shown (Roberts. et al 2002).

$$HO{-}(CH_2CH_2O)_n{-}CH_2CH_2{-}OH$$

Coupling of PEG has many advantages: it is non-toxic, non-antigenic, high soluble in water. It is also FDA approved. In detail, PEG conjugation shields antigenic epitopes thus reducing clearance by immune system and proteolytic enzymes. PEG also increases the size thus reducing

renal filtration (Roberts. et al 2002, Veronese 2000). Its solubility in water could also improve the solubility of polypeptides, making PEG the enhancement of peptides and protein as therapeutic drugs.

Here we applied PEG12 following NR2B9C and the modified peptide became TAT-NR2B9C-PEG12-CMA. Also, since TAT-CMA already showed toxicity (data not shown here), we also linked PEG12 in between synthesizing TAT-PEG12-CMA. The toxicity was tested and results were shown in Figure3.10B. TAT-NR2B9C-PEG12-CMA also showed no toxicity in a dose response up to 25uM. Similarly, TAT-PEG12-CMA showed no toxicity either (data not shown here). A Spacer between TAT and CMA did help to reduce the biological interference.

Unfortunately, as for the knockdown effect, TAT-NR2B9C-PEG12-CMA seemed to lose the ability to knockdown PSD95 (Fig 3.10C).

Besides PEG12, we also tried other spacers such as four glutamates or four glycines in other peptides (Data not shown here). Four glycines didn't reduce the toxicity and four glutamates reduced the toxicity with reduced knockdown effect. Glutamate has negative charge that compromises the penetrating property of TAT. Thus, the addition of linker could efficiently reduce the toxicity of the peptides, but the knockdown effect was also compromised.

3.2.6 Short CMA targeting motif

Identifying that all the peptides with CMA showed toxicity in chapter 4.2.1, I reasoned that reducing the number of target motif may induce better feature for the peptides.

For the CMA targeting motif, it had been shown that one motif was enough for targeting (Dice 1990). But some proteins contained more than one CMA targeting motif which may be exposed in different conditions. The original idea that we linked three CMA targeting motifs together was to increase the possible recognition of hsc70. Since encountering the toxicity problem, linking one CMA targeting motif to the binding sequence may reduce toxicity, and at the same time maintain its lysosome targeting efficacy.

Another concern on the property of the peptide was that the binding sequence of NR2B to PSD95 located at the C-terminal of NR2B, but after linking the CMA targeting motif to it, NR2B9C was no longer at the C terminal. This may have influenced the binding affinity.

Thus I synthesized two new peptides TAT-NR2B9C-QREFK and KFERQ-TAT-NR2B9C with 25 amino acids, 9 less than the original TAT-NR2B9C-CMA. Compared to TAT-NR2B9C-CMA, both TAT-NR2B9C-QREFK and KFERQ-TAT-NR2B9C showed no toxicity up to 25uM (Figure 3.11E). There was no big difference between TAT-NR2B9C-QREFK and KFERQ-TAT-NR2B9C.

As for the knockdown effect, TAT-NR2B9C-QREFK was better than KFERQ-TAT-NR2B9C (Fig 3.11 C and D). Even 5uM worked well in knocking down PSD95 without causing toxicity. Thus, after the above detailed characterizations, I finally reached a conclusion that using short CMA targeting motif is the most promising way to reduce the toxicity while keeping the knockdown efficacy.

3.2.7 Modifications of TAT-Geph1-CMA

After determining that short CMA targeting motif and addition of linker contributed to the reduction of toxicity, I next applied the two strategies to TAT-Geph1-CMA that targeted gephyrin. First, I replaced the CMA with KFERQ with 27 amino acids compared to the original 36. LDH assay was done 24h after the treatment at different concentrations from 5uM to 25uM. As expected, TAT-Geph1-QREFK showed less toxicity than TAT-Geph1-CMA, though the toxicity was not eliminated completely (Fig 3.5 and Fig 3.12E). Next, I inserted a linker between Geph1 and QREFK and the modified peptide became TAT-Geph1-PEG12-QREFK. The toxicity was reduced further compared to TAT-Geph1-QREFK (Figure3.12E). The result confirmed that both strategies worked in reducing the toxicity. But in contrast to TAT-NR2B9C-CMA, the extent that the toxicity was reduced was not as good as TAT-NR2B9C-QREFK and TAT-NR2B9C-PEG12-CMA which showed no toxicity at all up to 25uM.

As for the knockdown efficacy, the two modified peptides for gephyrin were also tested. TAT-Geph1-QREFK showed significant knockdown at the concentration higher than 15uM (Figure3.12C) while TAT-Geph1-PEG12-QREFK did not show dramatic knockdown of gephyrin (Figure3.12D). Thus, consistent with the modifications to TAT-NR2B-CMA, shortening the CTM is effective in reducing toxicity without compromising knockdown efficacy, whereas addition of a linker may reduce toxicity, but also compromise knockdown efficacy.

Table 3.1 Mutated peptides derived from GlyR β398-408

FSIVGSLPRDF	FSILGSLPRDF	FSFIGSLPRDF	FKIVGSLPRDF	FKILGSLPRDF	FKFIGSLPRDF
FSIVGSLPRDW	FSILGSLPRDW	FSFIGSLPRDW	FKIVGSLPRDW	FKILGSLPRDW	FKFIGSLPRDW
FSIVGSLPRDI	FSILGSLPRDI	FSFIGSLPRDI	FKIVGSLPRDI	FKILGSLPRDI	FKFIGSLPRDI
FSIVGSLKRDF	FSILGSLKRDF	FSFIGSLKRDF	FKIVGSLKRDF	FKILGSLKRDF	FKFIGSLKRDF
FSIVGSLKRDW	FSILGSLKRDW	FSFIGSLKRDW	FKIVGSLKRDW	FKILGSLKRDW	FKFIGSLKRDW
FSIVGSLKRDI	FSILGSLKRDI	FSFIGSLKRDI	FKIVGSLKRDI	FKILGSLKRDI	FKFIGSLKRDI
FSIVGSLVRDF	FSILGSLVRDF	FSFIGSLVRDF	FKIVGSLVRDF	FKILGSLVRDF	FKFIGSLVRDF
FSIVGSLVRDW	FSILGSLVRDW	FSFIGSLVRDW	FKIVGSLVRDW	FKILGSLVRDW	FKFIGSLVRDW
FSIVGSLVRDI	FSILGSLVRDI	FSFIGSLVRDI	FKIVGSLVRDI	FKILGSLVRDI	FKFIGSLVRDI
FSIVGSFPRDF	FSILGSFPRDF	FSFIGSFPRDF	FKIVGSFPRDF	FKILGSFPRDF	FKFIGSFPRDF
FSIVGSFPRDW	FSILGSFPRDW	FSFIGSFPRDW	FKIVGSFPRDW	FKILGSFPRDW	FKFIGSFPRDW
FSIVGSFPRDI	FSILGSFPRDI	FSFIGSFPRDI	FKIVGSFPRDI	FKILGSFPRDI	FKFIGSFPRDI
FSIVGSFKRDF	FSILGSFKRDF	FSFIGSFKRDF	FKIVGSFKRDF	FKILGSFKRDF	FKFIGSFKRDF
FSIVGSFKRDW	FSILGSFKRDW	FSFIGSFKRDW	FKIVGSFKRDW	FKILGSFKRDW	FKFIGSFKRDW
FSIVGSFKRDI	FSILGSFKRDI	FSFIGSFKRDI	FKIVGSFKRDI	FKILGSFKRDI	FKFIGSFKRDI
FSIVGSFVRDF	FSILGSFVRDF	FSFIGSFVRDF	FKIVGSFVRDF	FKILGSFVRDF	FKFIGSFVRDF
FSIVGSFVRDW	FSILGSFVRDW	FSFIGSFVRDW	FKIVGSFVRDW	FKILGSFVRDW	FKFIGSFVRDW
FSIVGSFVRDI	FSILGSFVRDI	FSFIGSFVRDI	FKIVGSFVRDI	FKILGSFVRDI	FKFIGSFVRDI
FSIVASLPRDF	FSILASLPRDF	FSFIASLPRDF	FKIVASLPRDF	FKILASLPRDF	FKFIASLPRDF
FSIVASLPRDW	FSILASLPRDW	FSFIASLPRDW	FKIVASLPRDW	FKILASLPRDW	FKFIASLPRDW
FSIVASLPRDI	FSILASLPRDI	FSFIASLPRDI	FKIVASLPRDI	FKILASLPRDI	FKFIASLPRDI
FSIVASLKRDF	FSILASLKRDF	FSFIASLKRDF	FKIVASLKRDF	FKILASLKRDF	FKFIASLKRDF
FSIVASLKRDW	FSILASLKRDW	FSFIASLKRDW	FKIVASLKRDW	FKILASLKRDW	FKFIASLKRDW
FSIVASLKRDI	FSILASLKRDI	FSFIASLKRDI	FKIVASLKRDI	FKILASLKRDI	FKFIASLKRDI
FSIVASLVRDF	FSILASLVRDF	FSFIASLVRDF	FKIVASLVRDF	FKILASLVRDF	FKFIASLVRDF
FSIVASLVRDW	FSILASLVRDW	FSFIASLVRDW	FKIVASLVRDW	FKILASLVRDW	FKFIASLVRDW
FSIVASLVRDI	FSILASLVRDI	FSFIASLVRDI	FKIVASLVRDI	FKILASLVRDI	FKFIASLVRDI
FSIVASFPRDF	FSILASFPRDF	FSFIASFPRDF	FKIVASFPRDF	FKILASFPRDF	FKFIASFPRDF
FSIVASFPRDW	FSILASFPRDW	FSFIASFPRDW	FKIVASFPRDW	FKILASFPRDW	FKFIASFPRDW
FSIVASFPRDI	FSILASFPRDI	FSFIASFPRDI	FKIVASFPRDI	FKILASFPRDI	FKFIASFPRDI
FSIVASFKRDF	FSILASFKRDF	FSFIASFKRDF	FKIVASFKRDF	FKILASFKRDF	FKFIASFKRDF
FSIVASFKRDW	FSILASFKRDW	FSFIASFKRDW	FKIVASFKRDW	FKILASFKRDW	FKFIASFKRDW
FSIVASFKRDI	FSILASFKRDI	FSFIASFKRDI	FKIVASFKRDI	FKILASFKRDI	FKFIASFKRDI
FSIVASFVRDF	FSILASFVRDF	FSFIASFVRDF	FKIVASFVRDF	FKILASFVRDF	FKFIASFVRDF
FSIVASFVRDW	FSILASFVRDW	FSFIASFVRDW	FKIVASFVRDW	FKILASFVRDW	FKFIASFVRDW
FSIVASFVRDI	FSILASFVRDI	FSFIASFVRDI	FKIVASFVRDI	FKILASFVRDI	FKFIASFVRDI

Table 3.1 Mutated peptides derived from GlyR β398-408 (Continued)

FSIIGSLPRDF	FSFVGSLPRDF	FSFLGSLPRDF	FKIIGSLPRDF	FKFVGSLPRDF	FKFLGSLPRDF
FSIIGSLPRDW	FSFVGSLPRDW	FSFLGSLPRDW	FKIIGSLPRDW	FKFVGSLPRDW	FKFLGSLPRDW
FSIIGSLPRDI	FSFVGSLPRDI	FSFLGSLPRDI	FKIIGSLPRDI	FKFVGSLPRDI	FKFLGSLPRDI
FSIIGSLKRDF	FSFVGSLKRDF	FSFLGSLKRDF	FKIIGSLKRDF	FKFVGSLKRDF	FKFLGSLKRDF
FSIIGSLKRDW	FSFVGSLKRDW	FSFLGSLKRDW	FKIIGSLKRDW	FKFVGSLKRDW	FKFLGSLKRDW
FSIIGSLKRDI	FSFVGSLKRDI	FSFLGSLKRDI	FKIIGSLKRDI	FKFVGSLKRDI	FKFLGSLKRDI
FSIIGSLVRDF	FSFVGSLVRDF	FSFLGSLVRDF	FKIIGSLVRDF	FKFVGSLVRDF	FKFLGSLVRDF
FSIIGSLVRDW	FSFVGSLVRDW	FSFLGSLVRDW	FKIIGSLVRDW	FKFVGSLVRDW	FKFLGSLVRDW
FSIIGSLVRDI	FSFVGSLVRDI	FSFLGSLVRDI	FKIIGSLVRDI	FKFVGSLVRDI	FKFLGSLVRDI
FSIIGSFPRDF	FSFVGSFPRDF	FSFLGSFPRDF	FKIIGSFPRDF	FKFVGSFPRDF	FKFLGSFPRDF
FSIIGSFPRDW	FSFVGSFPRDW	FSFLGSFPRDW	FKIIGSFPRDW	FKFVGSFPRDW	FKFLGSFPRDW
FSIIGSFPRDI	FSFVGSFPRDI	FSFLGSFPRDI	FKIIGSFPRDI	FKFVGSFPRDI	FKFLGSFPRDI
FSIIGSFKRDF	FSFVGSFKRDF	FSFLGSFKRDF	FKIIGSFKRDF	FKFVGSFKRDF	FKFLGSFKRDF
FSIIGSFKRDW	FSFVGSFKRDW	FSFLGSFKRDW	FKIIGSFKRDW	FKFVGSFKRDW	FKFLGSFKRDW
FSIIGSFKRDI	FSFVGSFKRDI	FSFLGSFKRDI	FKIIGSFKRDI	FKFVGSFKRDI	FKFLGSFKRDI
FSIIGSFVRDF	FSFVGSFVRDF	FSFLGSFVRDF	FKIIGSFVRDF	FKFVGSFVRDF	FKFLGSFVRDF
FSIIGSFVRDW	FSFVGSFVRDW	FSFLGSFVRDW	FKIIGSFVRDW	FKFVGSFVRDW	FKFLGSFVRDW
FSIIGSFVRDI	FSFVGSFVRDI	FSFLGSFVRDI	FKIIGSFVRDI	FKFVGSFVRDI	FKFLGSFVRDI
FSIIASLPRDF	FSFVASLPRDF	FSFLASLPRDF	FKIIASLPRDF	FKFVASLPRDF	FKFLASLPRDF
FSIIASLPRDW	FSFVASLPRDW	FSFLASLPRDW	FKIIASLPRDW	FKFVASLPRDW	FKFLASLPRDW
FSIIASLPRDI	FSFVASLPRDI	FSFLASLPRDI	FKIIASLPRDI	FKFVASLPRDI	FKFLASLPRDI
FSIIASLKRDF	FSFVASLKRDF	FSFLASLKRDF	FKIIASLKRDF	FKFVASLKRDF	FKFLASLKRDF
FSIIASLKRDW	FSFVASLKRDW	FSFLASLKRDW	FKIIASLKRDW	FKFVASLKRDW	FKFLASLKRDW
FSIIASLKRDI	FSFVASLKRDI	FSFLASLKRDI	FKIIASLKRDI	FKFVASLKRDI	FKFLASLKRDI
FSIIASLVRDF	FSFVASLVRDF	FSFLASLVRDF	FKIIASLVRDF	FKFVASLVRDF	FKFLASLVRDF
FSIIASLVRDW	FSFVASLVRDW	FSFLASLVRDW	FKIIASLVRDW	FKFVASLVRDW	FKFLASLVRDW
FSIIASLVRDI	FSFVASLVRDI	FSFLASLVRDI	FKIIASLVRDI	FKFVASLVRDI	FKFLASLVRDI
FSIIASFPRDF	FSFVASFPRDF	FSFLASFPRDF	FKIIASFPRDF	FKFVASFPRDF	FKFLASFPRDF
FSIIASFPRDW	FSFVASFPRDW	FSFLASFPRDW	FKIIASFPRDW	FKFVASFPRDW	FKFLASFPRDW
FSIIASFPRDI	FSFVASFPRDI	FSFLASFPRDI	FKIIASFPRDI	FKFVASFPRDI	FKFLASFPRDI
FSIIASFKRDF	FSFVASFKRDF	FSFLASFKRDF	FKIIASFKRDF	FKFVASFKRDF	FKFLASFKRDF
FSIIASFKRDW	FSFVASFKRDW	FSFLASFKRDW	FKIIASFKRDW	FKFVASFKRDW	FKFLASFKRDW
FSIIASFKRDI	FSFVASFKRDI	FSFLASFKRDI	FKIIASFKRDI	FKFVASFKRDI	FKFLASFKRDI
FSIIASFVRDF	FSFVASFVRDF	FSFLASFVRDF	FKIIASFVRDF	FKFVASFVRDF	FKFLASFVRDF
FSIIASFVRDW	FSFVASFVRDW	FSFLASFVRDW	FKIIASFVRDW	FKFVASFVRDW	FKFLASFVRDW
FSIIASFVRDI	FSFVASFVRDI	FSFLASFVRDI	FKIIASFVRDI	FKFVASFVRDI	FKFLASFVRDI

Table 3.2 Peptides that showed positive binding to GephE in the first round of high density peptide array

FSIVGSLPRDF	FSIVGSLVRDW	FSIVGSFKRDI	FSIIGSLKRDF	FSIIGSFPRDW	FSIIGSFVRDI
FSIVGSLPRDW	FSIVGSLVRDI	FSIVGSFVRDF	FSIIGSLKRDW	FSIIGSFPRDI	FSILGSLPRDF
FSIVGSLPRDI	FSIVGSFPRDF	FSIVGSFVRDW	FSIIGSLKRDI	FSIIGSFKRDF	FSILGSFPRDF
FSIVGSLKRDF	FSIVGSFPRDW	FSIVGSFVRDI	FSIIGSLVRDF	FSIIGSFKRDW	FKIVGSLPRDF
FSIVGSLKRDW	FSIVGSFPRDI	FSIIGSLPRDF	FSIIGSLVRDW	FSIIGSFKRDI	FKIVGSFPRDF
FSIVGSLKRDI	FSIVGSFKRDF	FSIIGSLPRDW	FSIIGSLVRDI	FSIIGSFVRDF	
FSIVGSLVRDF	FSIVGSFKRDW	FSIIGSLPRDI	FSIIGSFPRDF	FSIIGSFVRDW	

Table 3.3 Peptides that showed consistent binding to GephE after two rounds of screening

FSIVGSLPRDF	FSIVGSLPRDI	FSIVGSFPRDF	FSIIGSLPRDF	FSIIGSLPRDI

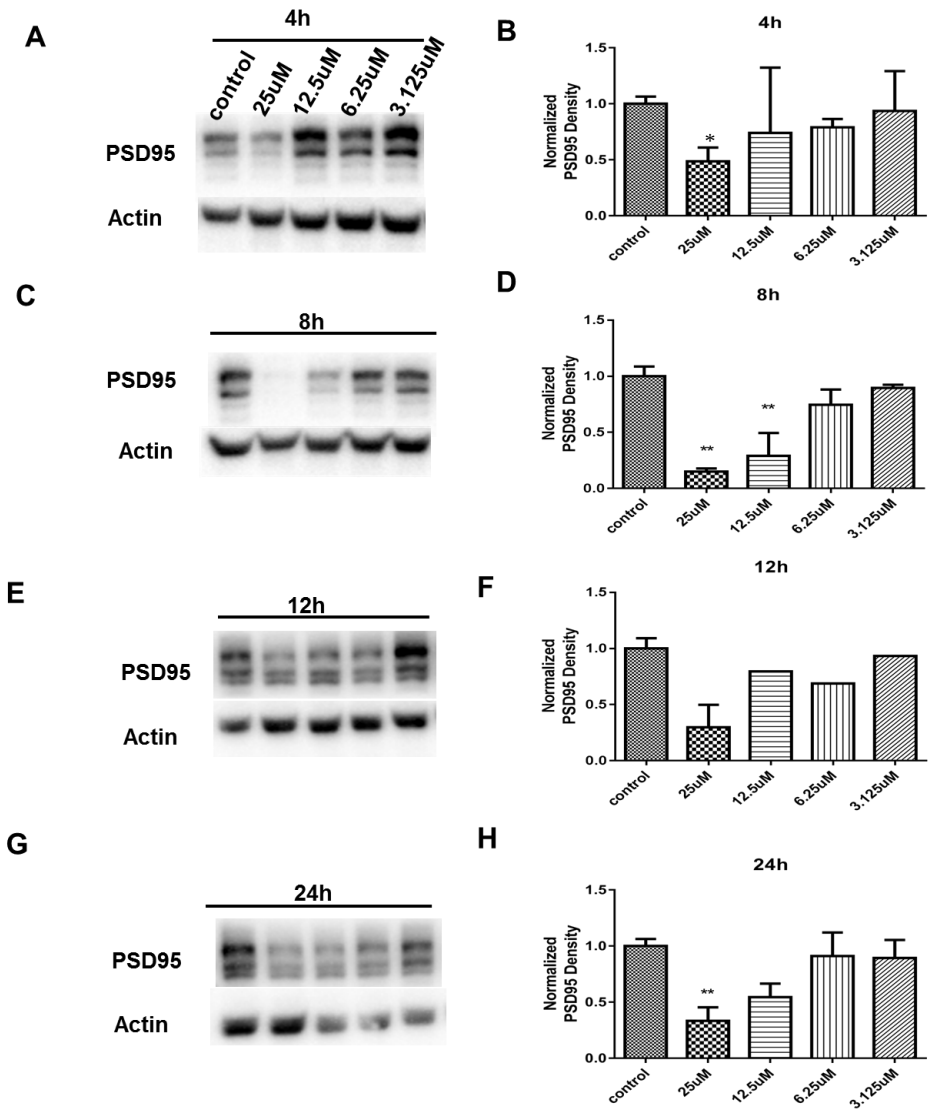

Figure 3.1Time and dose response of TAT-NR2B9C-CMA on Knocking down PSD95

Immunoblots sequentially probed for PSD95 and β-actin (as the loading control) showing the knockdown of PSD95 mediated by TAT-NR2B9C-CMA. **A**: The representative of immunoblots when the cells were treated for 4h; **B**: The statistical analysis was done and the knockdown showed a dose dependent way (n=3). At 25uM, PSD95 was knocked down to 48%. Similarly, **C** and **D** showed the immunoblots when the cells were treated for 8h (n=3) and **E** and **F** showed the result of 12h (it is done once for a quick test) and **G** and **H** showed the result of 24h. The statistical analysis was determined by one way ANOVA. Data shown as means ± SEM. *p<0.05, **p<0.01 compared with control.

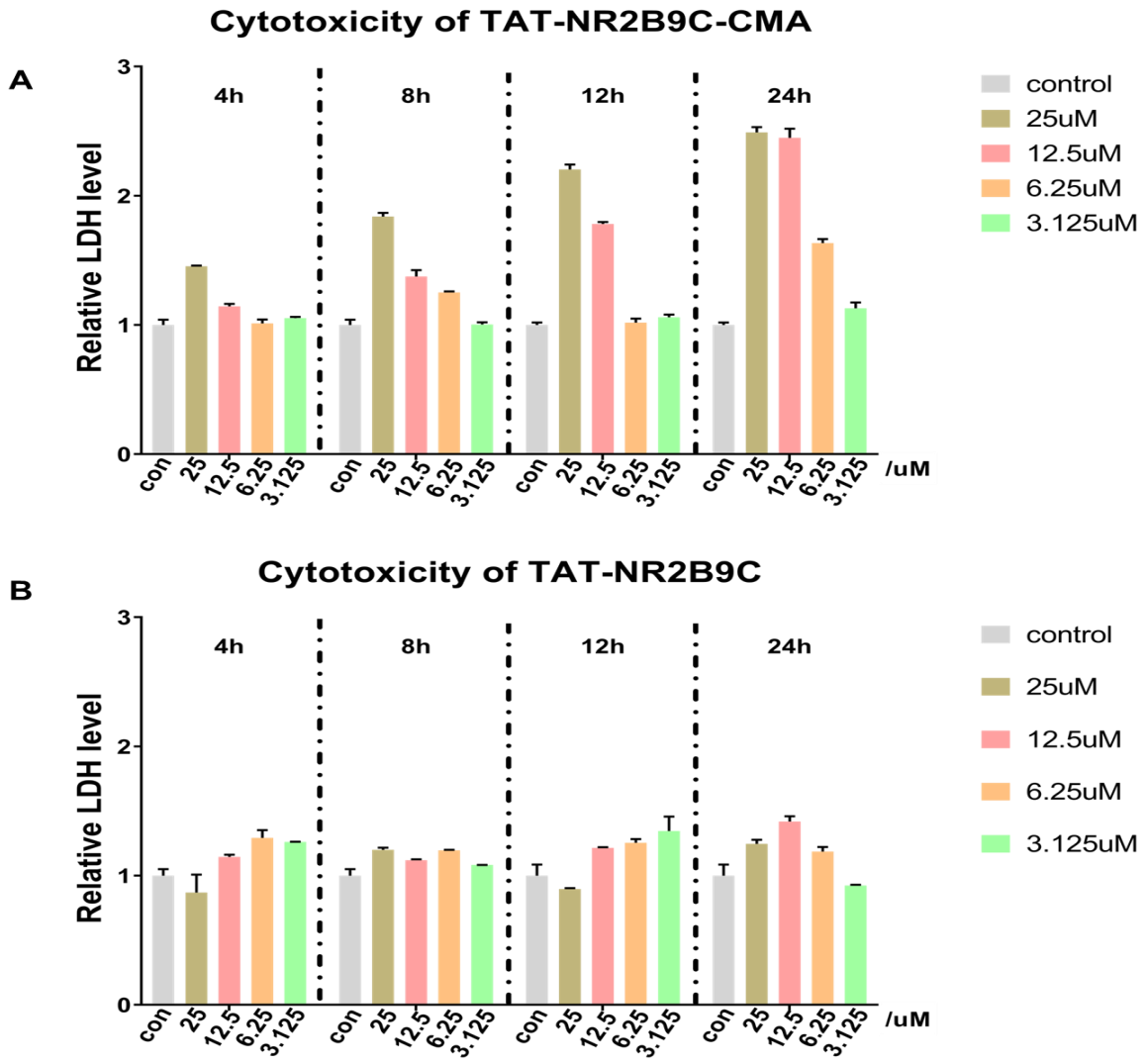

Figure 3.2 Cytotoxicity of TAT-NR2B9C-CMA and TAT-NR2B9C

A: LDH assay was applied to test the toxicity of TAT-NR2B9C-CMA. The media were collected after the cells were treated for 4h, 8h, 12h and 24h and the toxicity showed a time and dose dependent way. After 8h, LDH was almost twice as control at 25uM. After 12h, LDH at 12.5uM and 25uM increased more. After 24h, the LDH was similar between 12.5uM and 25uM, which were both around 2.5 times compared to control. And LDH went up even at 6.25uM. **B**: LDH assay were also done for TAT-NR2B9C as control. TAT-NR2B9C showed no toxicity within those time and concentration range. Those data are from two repeats of experiments.

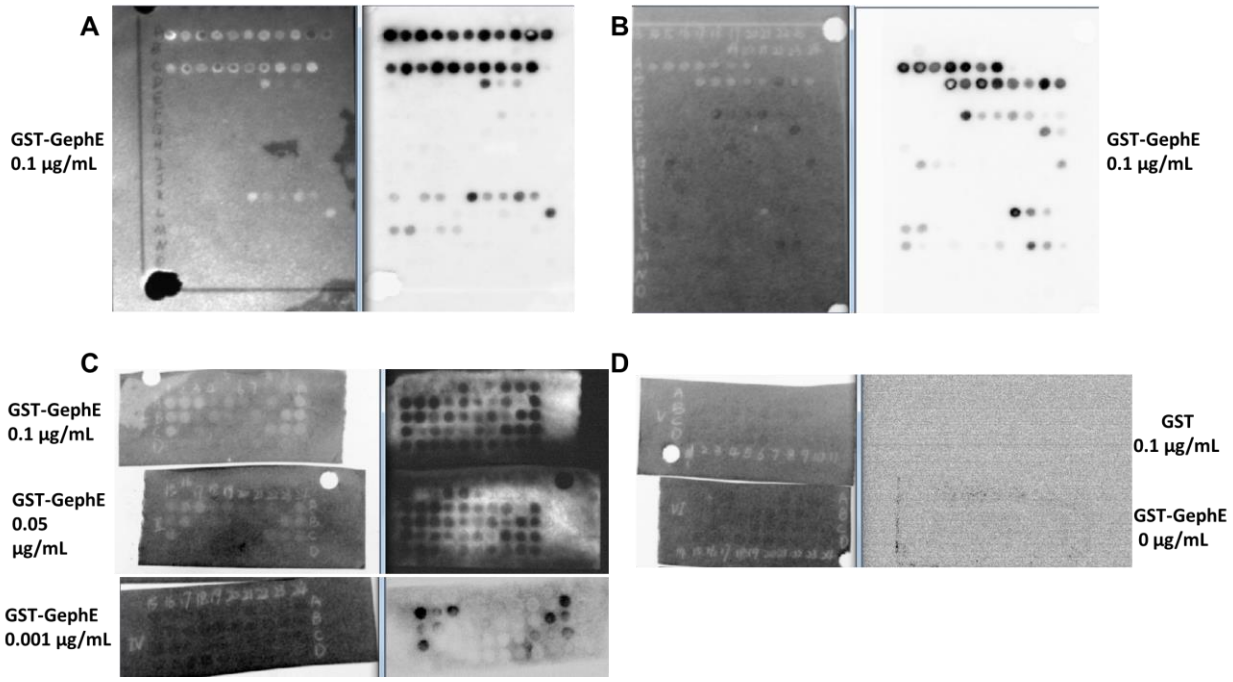

Figure 3.3 Screening of peptides for gephyrin using high density peptide arrays

Purified recombinant protein GST-GephE was used to overlay with peptide arrays. **A** and **B**: First round of screening of the peptides. 432 peptides were synthesized on the filter paper and each dot represented one. The membrane was probed with anti-gephyrin antibody after it was incubated with GST-GephE at the concentration of 0.1ug/mL. Each dark dot represented a positive peptide that would bind with GST-GephE. **C**: Second round of screening using those showed binding in the first round of screening. The membranes were incubated with GST-GephE at a series of concentrations: 0.1ug.mL, 0.05ug/mL and 0.001ug/mL and probed with anti-gephyrin antibody. **D**: Control membranes probed with anti-gephyrin antibody incubated with purified GST protein or in absence of GST-GephE. Those screening data is from one experiment.

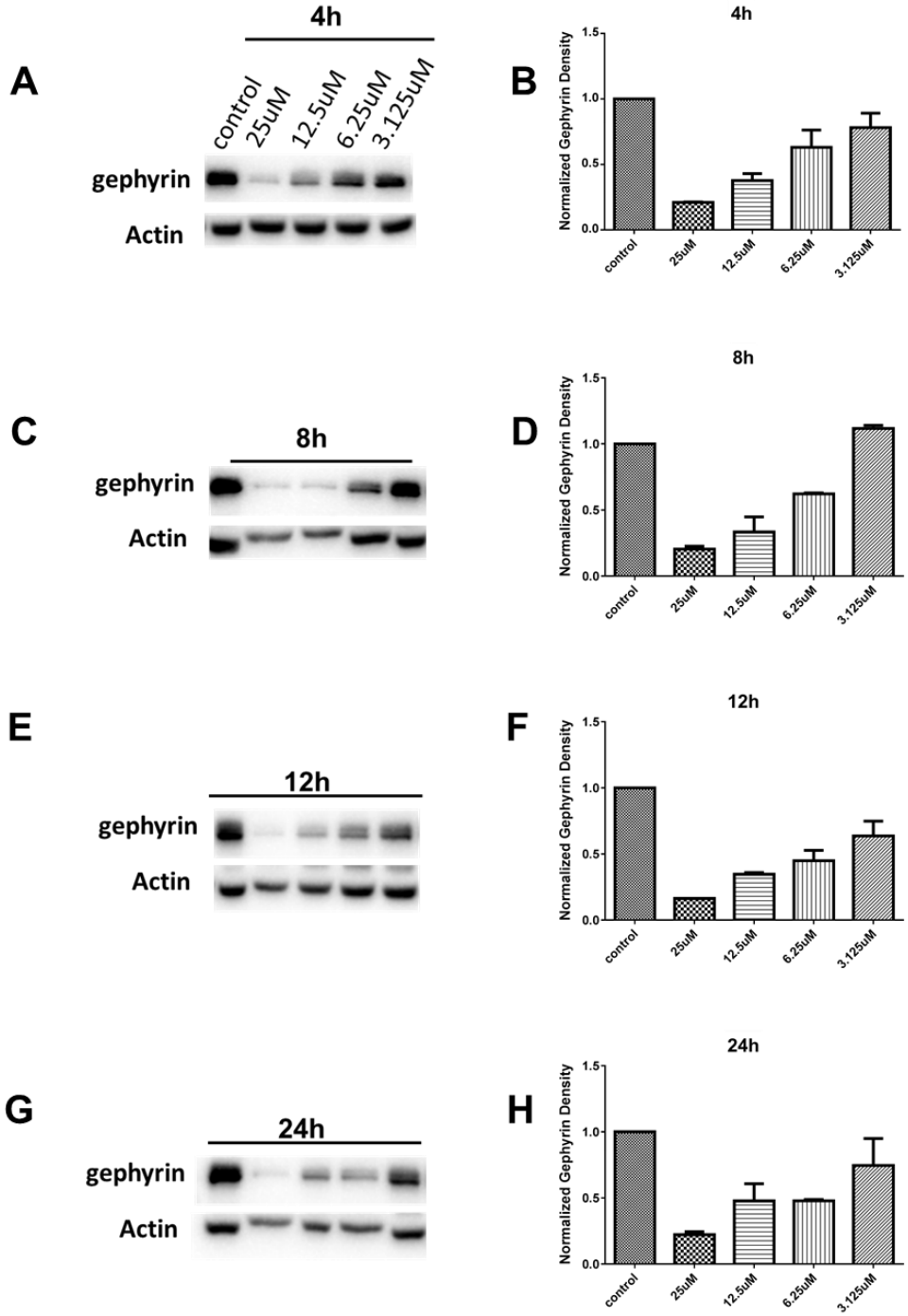
A
4h
control
25uM
12.5uM
6.25uM
3.125uM
gephyrin
Actin
B
4h
Normalized Gephyrin Density
1.5
1.0
0.5
0.0
control
25uM
12.5uM
6.25uM
3.125uM
C
8h
gephyrin
Actin
D
8h
Normalized Gephyrin Density
E
12h
gephyrin
Actin
F
12h
Normalized Gephyrin Density
G
24h
gephyrin
Actin
H
24h
Normalized Gephyrin Density

Figure 3.4 Time and dose response of TAT-Geph1-CMA on Knocking down gepyrin

Immunoblots sequentially probed for gephyrin and β-actin showing the knockdown of gephyrin mediated by TAT-Geph1-CMA. **A**: The representative of immunoblots when the cells were treated for 4h; **B**: The analysis was done and the knockdown showed a dose dependent way at 4h. At 25uM, only 21 ± 0.4% of gephyrin was left. At 12.5uM, 37 ±5.3% was left. At 6.25uM and 3.125uM, the number became 62.9 ±13.3% and 78.1 ±18.1% respectively. Similarly, **C** and **D** showed the immunoblots when the cells were treated for 8h and **E** and **F** showed the result of 12h and **G** and **H** showed the result of 24h. The knockdown had the same trend after different period of treatments. The experiments were repeated twice, but the trend was quite clear.

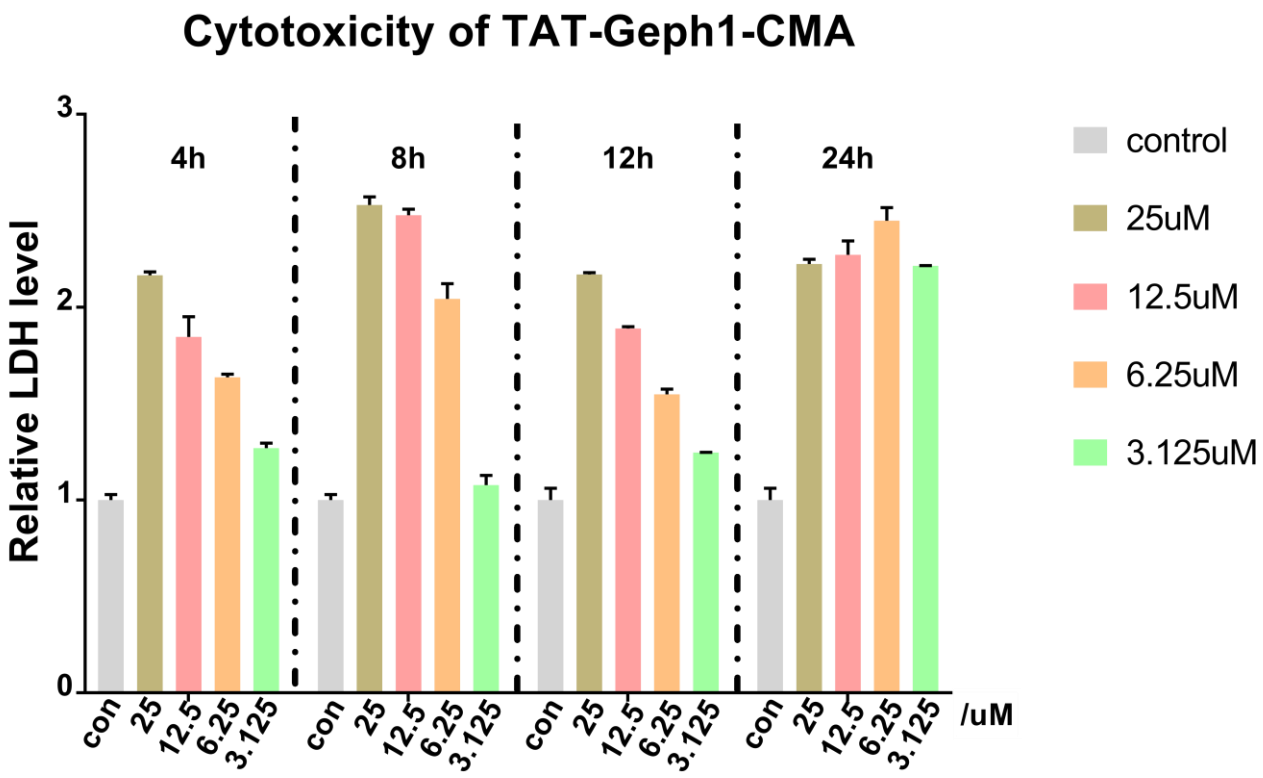

Figure 3.5 Cytotoxicity of TAT-Geph1-CMA

LDH assay was applied to test the toxicity of TAT-Geph1-CMA. The media were collected after the cells were treated for 4h, 8h, 12h and 24h and the toxicity showed a time and dose dependent way. After 4h, the peptide started to show toxicity when the concentration was higher than 6.25uM. At 8h and 12h, the LDH increased more and at 24h, the LDH level was similar between 6.25uM, 12.5uM and 25uM and the LDH went up even at 3.125uM. Those data are from two repeats of experiments.

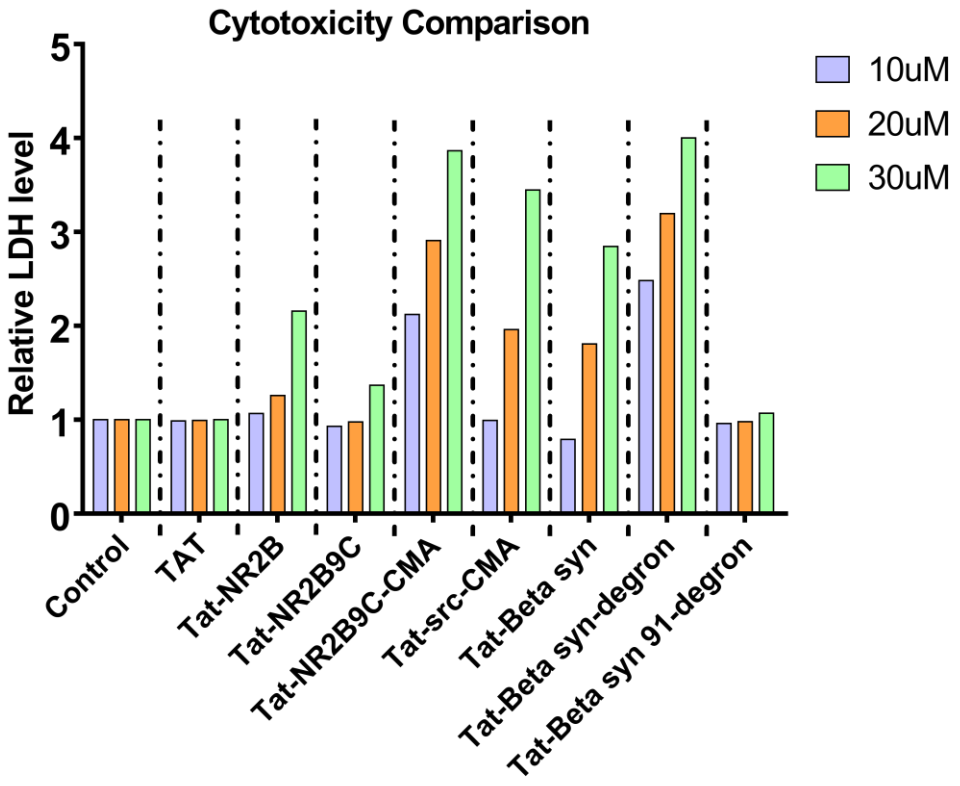

Figure 3.6 Identifying cytotoxicity by comparing different peptides

LDH assay was applied to test the toxicity of different peptides. The media were collected after the cells were treated 24h at the concentrations of 10uM, 20uM and 30uM. TAT showed no toxicity up to 30uM. TAT-NR2B showed no toxicity up to 20uM and at 30uM, LDH was twice as much as control. TAT-NR2B9C showed no toxicity. TAT-NR2B9C-CMA showed toxicity at 10uM, 20uM and 30uM. TAT-src-CMA showed toxicity at 20uM, 30uM but not 10uM. Those data shown here is from one experiment but it is repeated by others (Data not shown here).

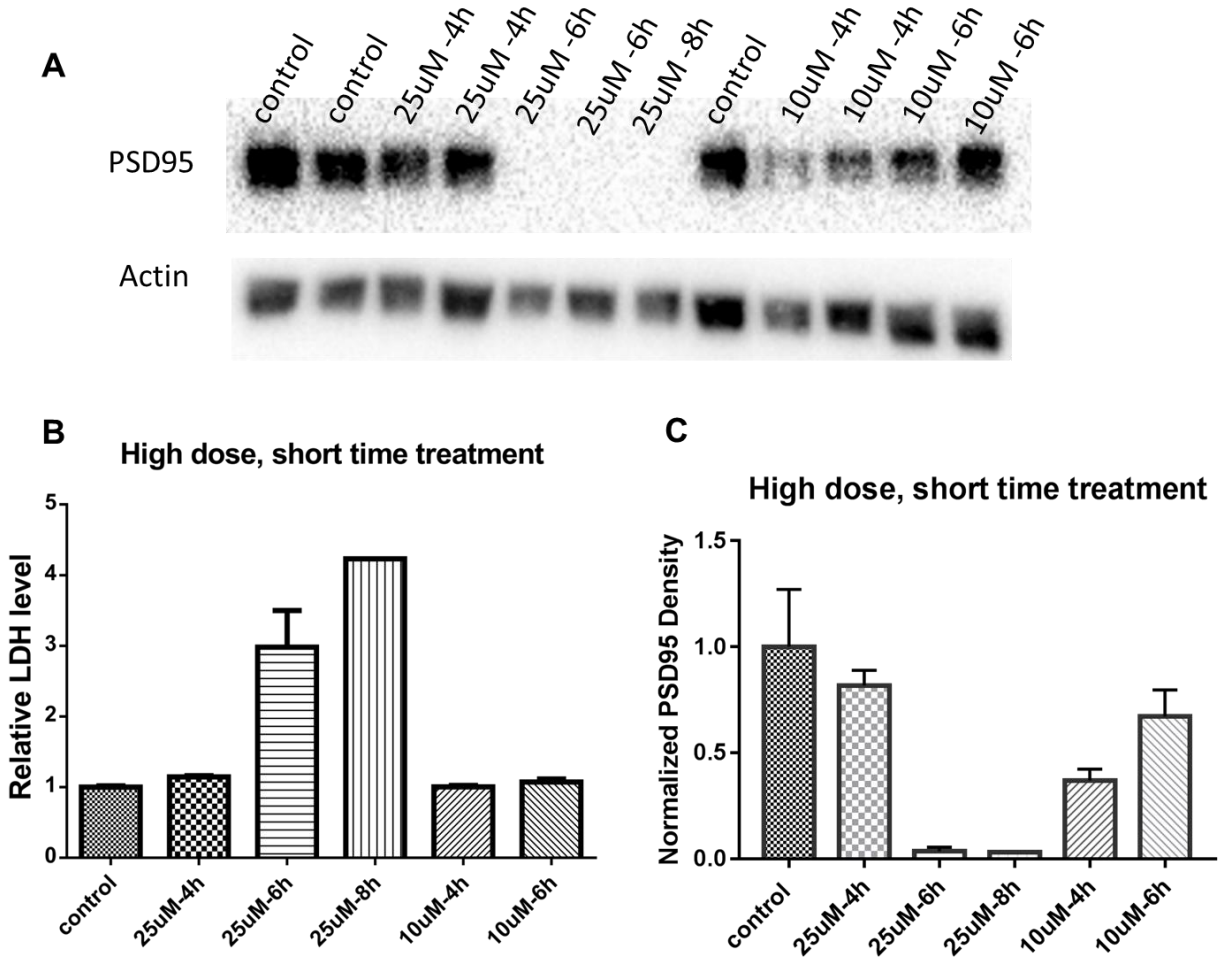

Figure 3.7 Cytotoxicity and efficacy test using protocol with high dose, short time treatment

Neurons were treated with TAT-NR2B9C-CMA at 10uM or 25uM for 2h and the peptide was washed away. Media and neurons were collected after the desired time of waiting. **A**: Representative of immunoblots probed with anti-PSD95 antibody. **B**: LDH analysis showed the cytotoxicity. Neurons showed toxicity three or four times as control after waiting for more than 6h following being exposed to the peptide for 2h. At 10uM, the protocol seemed to eliminate the toxicity. **C**: Analysis of the knockdown efficacy. PSD95 was almost gone after waiting for more than 6h following being exposed to the peptide for 2h. At 10uM, more than 50% of PSD95 was knocked down. N=2

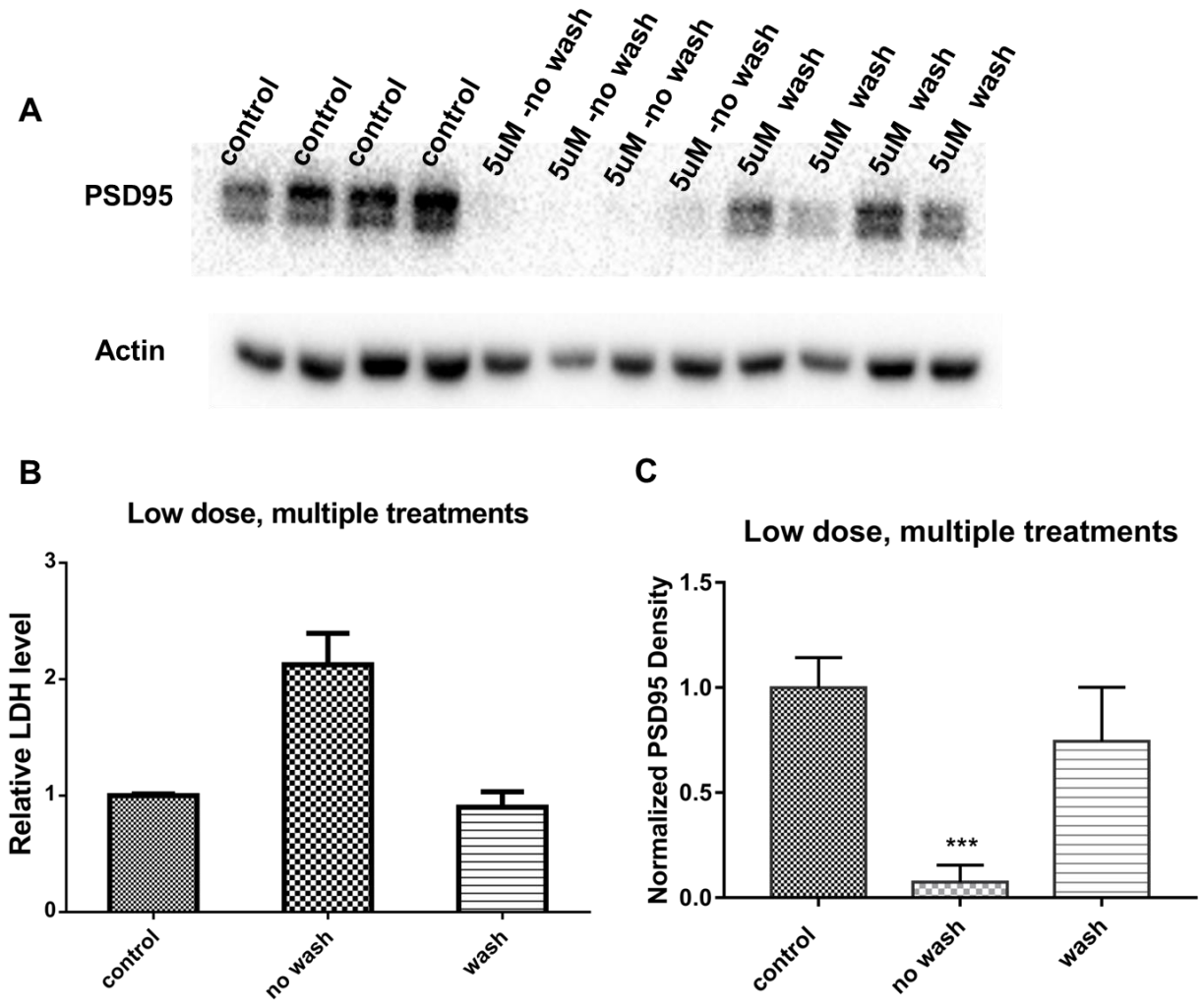

Figure 3.8 Cytotoxicity and efficacy test using protocol with low dose, multiple treatments

Neurons were treated with TAT-NR2B9C-CMA at 5uM and in every two hours, the peptide was washed away or not followed a new batch of 5uM TAT-NR2B9C-CMA was added to the media. For the no wash protocol, the peptide would accumulate while for the wash protocol, the concentration of peptide would not be higher than 5uM. Media and neurons were collected after 5 cycles which meant 10h. **A**: Representative of immunoblots probed with anti-PSD95 antibody. **B**: LDH analysis showed the cytotoxicity. Neurons showed toxicity in no wash protocol but not in wash protocol. **C**: Analysis of the knockdown efficacy. PSD95 was almost gone for the no wash protocol while there was 35% of knockdown in the wash protocol. The statistical analysis (N=3) was determined by one way ANOVA. Data shown as means ± SEM. *p<0.05, **p<0.01, ***p<0.001 compared with control.

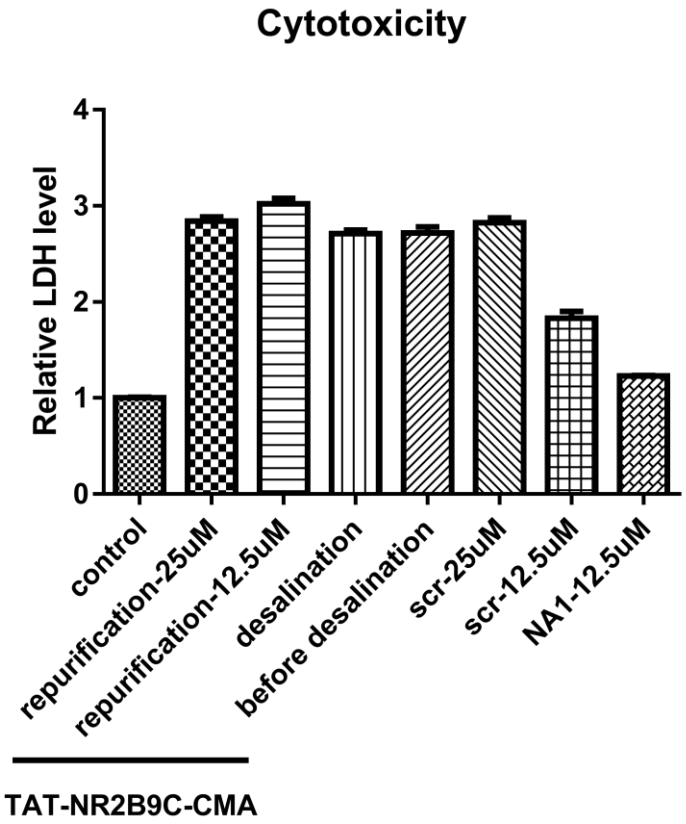

Figure 3.9 Cytotoxicity of TAT-NR2B9C-CMA after modifying purification procedure

LDH assay was applied to test the toxicity after modifying the purification procedure. The one before desalination equaled to the original one. Neither repurification nor desalination reduced the LDH level. The cytotoxicity of TAT-NRB9C-CMA was not due to the chemicals contained in the synthesis nor the purity as it reached more than 95% after the original synthesis. The purity would reach more than 99% after repurification which didn't contribute to reduce the cytotoxicity. Those data are from two repeats of experiments.

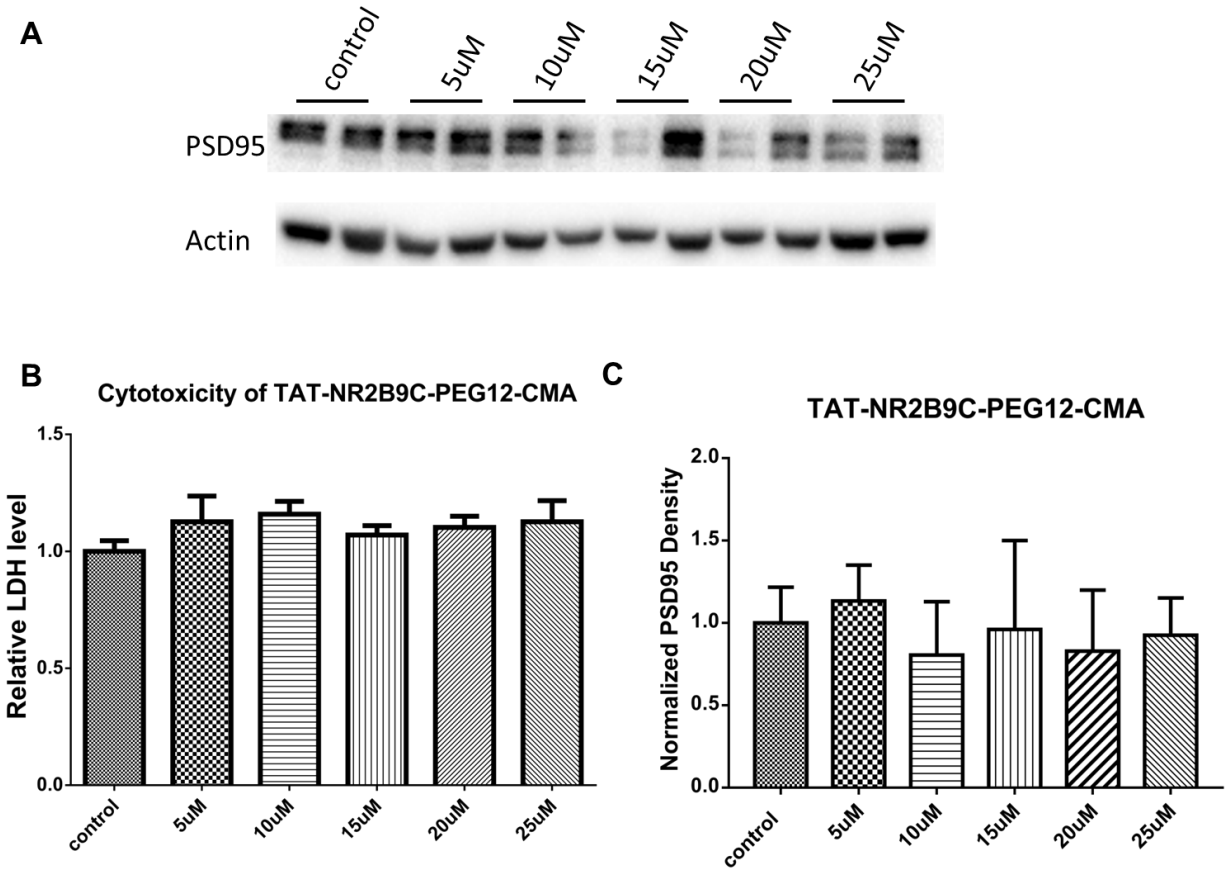

Figure 3.10 Cytotoxicity and efficacy test after addition of linker

TAT-NR2B9C-CMA was modified by adding a PEG12 linker between NR2B9C and CMA as a spacer. The neurons were treated with TAT-NR2B9C-PEG12-CMA for 24h at the concentrations of 5uM, 10uM, 15uM, 20uM and 25uM respectively. **A**: Representative of immunoblots sequentially probed with anti-PSD95 and anti-β-actin antibody. **B**: LDH analysis showed the cytotoxicity. Neurons showed no toxicity at those concentrations. **C**: Analysis of the knockdown efficacy. PSD95 was not knocked down by TAT-NR2B9C-PEG12-CMA. The statistical analysis was determined by one way ANOVA. Data shown as means ± SEM (n=4). *p<0.05, **p<0.01, ***p<0.001 compared with control.

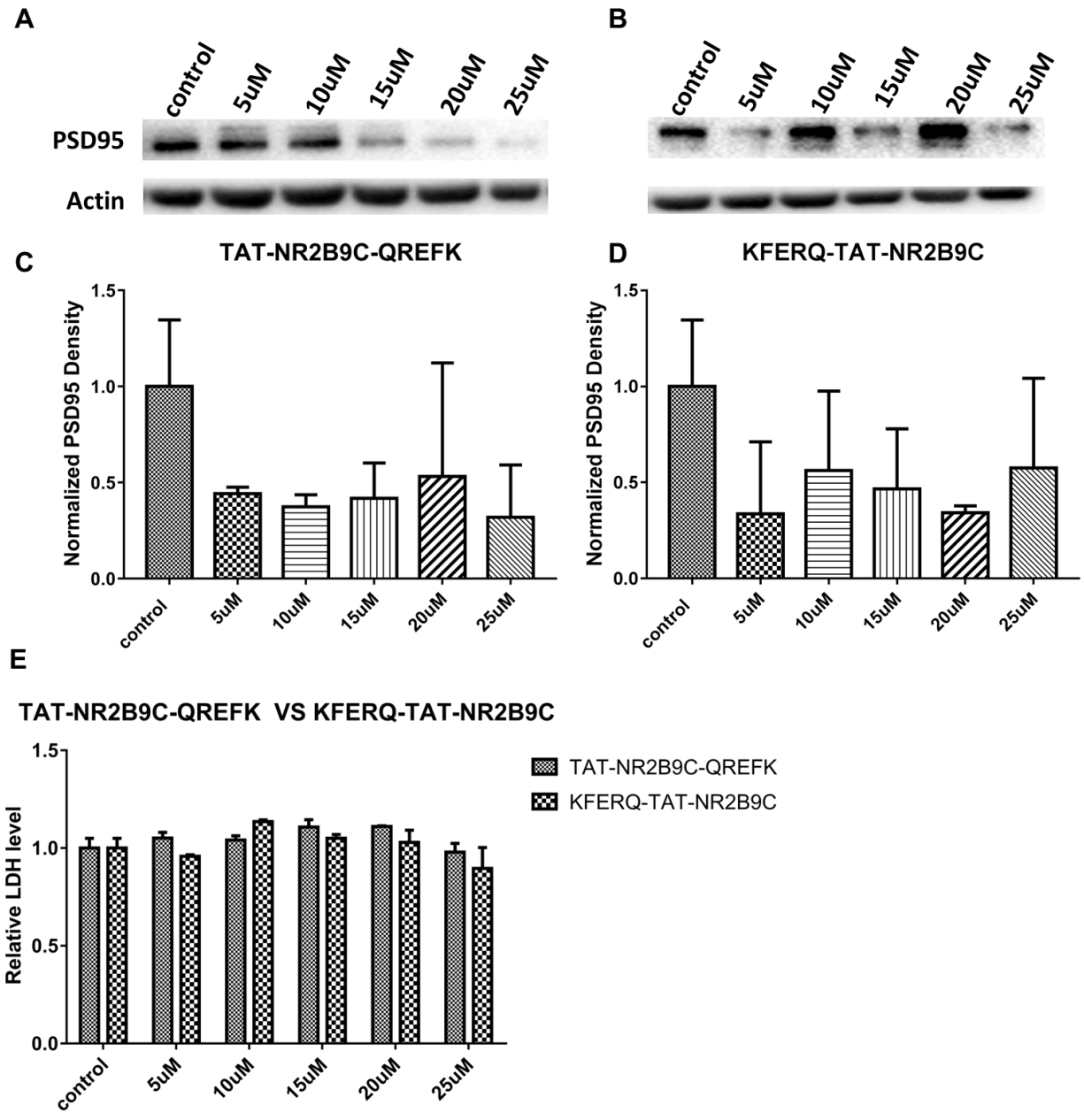

Figure 3.11 Cytotoxicity and efficacy test after using short CMA targeting motif

Short CMA targeting motif replaced the original 14aa one. And NR2B9C was also placed at the C-terminal to see whether it had a better binding. The neurons were treated with TAT-NR2B9C-QREFK or KFERQ-TAT-NR2B9C for 24h at the concentrations of 5uM, 10uM, 15uM, 20uM and 25uM respectively. **A**: Representative of immunoblots sequentially probed with anti-PSD95 and anti-β-actin antibody after treating with TAT-NR2B9C-

QREFK. **B**: Representative of immunoblots sequentially probed with anti-PSD95 and anti-β-actin antibody after treating with KFERQ-TAT-NR2B9C. **C**: Analysis of the knockdown efficacy of TAT-NR2B9C-QREFK. More than 50% of PSD95 was knocked down from 5uM. **D**: Analysis of the knockdown efficacy of KFERQ-TAT-NR2B9C. There was trend of PSD95 being knocked down but the variation was big. Data shown as means ± SEM. For concentration of 15uM and 25uM, n=3. Other concentrations were repeated twice. **E**: LDH assay was applied. Both TAT-NR2B9C-QREFK and KFERQ-TAT-NR2B9C showed no toxicity.

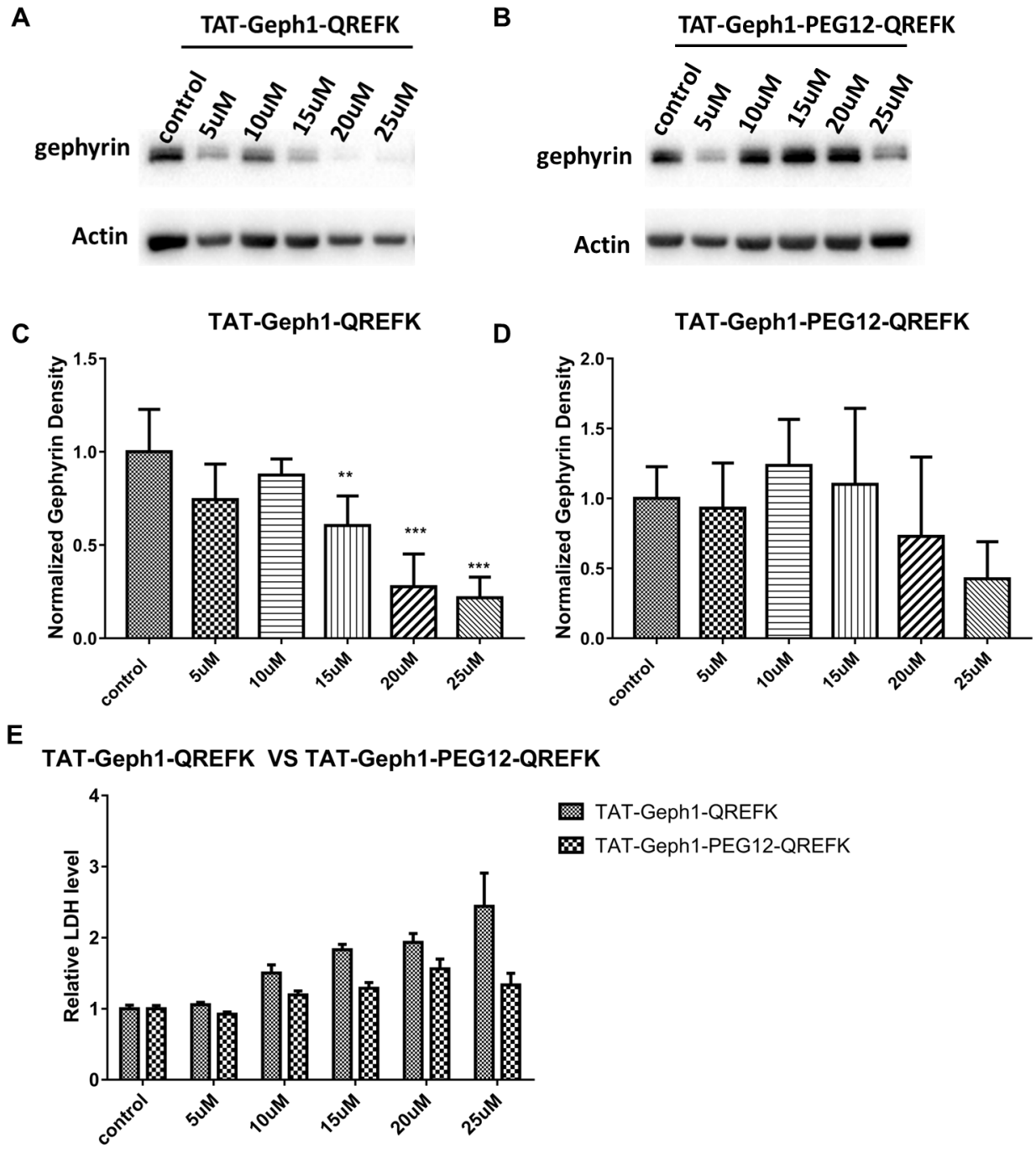

Figure 3.12 Modification of TAT-Geph1-CMA

TAT –Geph1-CMA was also modified. The neurons were treated with modified peptides for 24h at the concentrations of 5uM, 10uM, 15uM, 20uM and 25uM respectively. **A**: Representative of immunoblots sequentially

probed with anti-gephyrin and anti-β-actin antibody after treating with Geph1-QREFK. **B**: Representative of immunoblots sequentially probed with anti-gephyrin and anti-β-actin antibody after treating with Geph1-PEG12-QREFK. **C**: Analysis of the knockdown efficacy of TAT-Geph1-QREFK. Gephyrin was significantly knocked down at 15uM, 20uM and 25uM ($n=4$, $F(5,22)=15.81$, $p<0.001$). **D**: Analysis of the knockdown efficacy of TAT-Geph1-PEG12-QREFK. There was no significance here ($n=4$, $F(5,22)=2.45$, $p>0.05$). **E**: LDH assay was applied. TAT-Geph1-QREFK still showed some toxicity at the concentration of more than 15uM. TAT-Geph1-PEG12-QREFK showed no toxicity at those concentrations. The statistical analysis was determined by one way ANOVA. Data shown as means ± SEM ($n=4$). *$p<0.05$, **$p<0.01$, ***$p<0.001$ compared with control.

Chapter 4: Conclusion discussion and future directions

In this study, I set out to develop and optimize peptides that can specifically and reversibly knock down PSD95 or gephyrin via peptide mediated degradation. Our ultimate goal is to be able to use these peptides to modulate the excitatory and inhibitory balance in CNS by knocking down PSD95 or gephyrin since the excitatory and inhibitory synapses are closely interact with those scaffolding proteins. During the efficacy test of those peptides, I found that the knockdown effect was often associated with the cell death indicated by LDH assay. Thus I came up with multiple strategies trying to reduce the toxicity while keeping the knockdown effect. The following is a summary of what I found:

1) The peptide TAT-NR2B9C-CMA has been designed to target PSD95. The efficacy test showed that the knockdown was dose-dependent. At 25uM, even at 4h, only half of PSD95 was left. But as the time and doses increased, the extracellular LDH also increased a lot. The increased LDH may also contribute to the knock down of protein.

2) Two rounds of screening were conducted to search for a peptide that could bind with gephyrin. After the screening, five peptides were chosen as the positive candidates. Meanwhile, the original peptide TAT-Geph1-CMA was used to do the efficacy test. The extent of knockdown was also dose-dependent, but at different times, the difference of knockdown was not dramatic. Similarly, cell death was also observed as time and dose increased. The cell death might also affect the knockdown effect.

3) Observing the cytotoxicity of TAT-NR2B9C-CMA and TAT-Geph1-CMA, I tested some other peptides and found that the cell toxicity appears a common issue for most of peptides especially the ones with longer multiple CMA motifs. The toxicity is also sequence-dependent.

4) To reduce the cytotoxicity of peptides, I first changed the treatment protocol. The high dose and short time treatments worked at 10uM but not 25uM. For low dose with multiple treatments in no wash protocol, the PSD95 was efficiently knocked down, but cytotoxicity remained high. In wash away protocol, the cytotoxicity was low, but PSD95 was not knocked down. The changing treatment regimens appear not a promising way to separate knock down efficacy and cell toxicity.

5) Changes of treatment protocol could only be applied in vitro and the application was restricted. Then I modified the purification procedure to increase the purity or get rid of TFA in the synthesis procedure. But neither re-purification nor desalination significantly reduces cytotoxicity.

6) Addition of linker to space the three different parts of the peptides was able to eliminate the cytotoxicity up to 25uM for TAT-NR2B9C-PEG12-CMA. But unfortunately, the knockdown effect was also eliminated.

7) Using short CMA targeting motif was the most promising to reduce the cytotoxicity while maintaining the knockdown efficacy.

8) The strategies could also be applied to other peptides, but the effect may vary with depending on the peptides - the reduction of cytotoxicity could be predicted, but the extent might vary depending on the individual peptides.

My work further confirmed that the peptide-based protein knockdown technology developed in our lab is a very effective means to rapidly and reversibly control protein levels in a cell. But, I also discovered that most of the peptides have various degree excitotoxicity that may limit the application of the technology. The mechanism by which these peptides exert toxic effect on neurons remained unclear. One idea was that TAT conjugated peptide would penetrate the membranes via direct translocation.

The positively charged arginines develop electrostatic interaction with negatively charged glycoproteins on the membrane, and the disturbance forms a transient pore on the membrane for the peptide to directly translocate into the cells (Derossi et al 1996). As the peptides became longer, they may form secondary structure and "drill" a hole on the membrane, resulting in the leaking of cytosolic substances.

LDH was one of the substances leaking into the extracellular solution when the plasma membrane integrity is compromised as described in Chapter 2.4. But since the method detected the concentration of LDH in the media and the LDH would accumulate, the LDH data I saw at certain time just reflected how much LDH was released by then. Here came the question: for the high dose short time treatment and low dose and multiple treatments, the LDH level might be

disturbed by the procedure of washing away the peptide and adding new batch of peptide. For example, at 10uM, TAT-NR2B9C-CMA was washed away after 2h. Then after another waiting for 6h or 8h, I detected the knockdown but not the cytotoxicity. But it didn't mean that the protocol was safe for longer time since more LDH could accumulate later. Thus more approaches to check the status of the neurons should be applied such as MTT to depict a clear health condition of the neurons.

I also observed that for the efficacy test of TAT-NR2B9C-CMA, the knockdown was dose dependent but the influence of treatment time was not as clear. It seemed that at 8h, the PSD95 got the best knockdown at the concentration of 12.5uM but when the time reached 12h, the knockdown was not as dramatic as 8h. And the knockdown effect of TAT-Geph1-CMA was quite similar between those time points. Each protein has their own turnover rate and the knockdown pattern was not expected to be the same. After a certain point, it is possible that more proteins were synthesized than being degraded and the protein level was elevated.

To change the intrinsic properties of peptides, I tried to put a linker between the binding sequence and the CMA targeting motif as a spacer. The linker of PEG12 and E4 (4 glutamates) could significantly reduce the toxicity while G4 (4 glycines) could not reduce the toxicity. But the peptides with PEG12 and E4 as a linker could not induce efficient knockdown of PSD95, which was a little surprising. It showed that the linker did more than just a spacer since the function of the peptide was also compromised. But it is hard to conclude as I didn't know the structure of the new peptide. Originally when I designed the peptide, I thought the peptides could be divided into three parts and each part fulfills their function individually. But the fact that

PEG12 reduced both cytotoxicity and knockdown effect might reveal that the peptide was functioning as a whole and any change to it might generate a peptide with different characteristics and functionality.

When the normal 14aa CMA was switched to one 5aa motif, the knockdown effect was kept while the toxicity was reduced. Here TAT-NR2B9C-QREFK showed better knockdown effect than KFERQ-TAT-NR2B9C while KFERQ-TAT-NR2B9C still showed the trend of knockdown. More repeats should help clarify if the addition of CMA to either N or C terminals of the peptide affects peptide's knockdown efficacy. The effect of linker on different peptides was also different. For example, both PEG12 and the short CMA targeting motif QREFK could reduce the toxicity of TAT-NR2B9C-CMA completely. But switching from 14aa CTM to 5aa CTM could only reduce the cytotoxicity of TAT-Geph1-CMA to some extent. And adding a PEG12 linker between binding sequence and the 5aa CTM could further reduce the cytotoxicity. Comparing the modification for TAT-NR2B9C-CMA and TAT-Geph1-CMA, the modification has to be custom to individual peptides using various strategies I tested in the present work.

Here I did the different kinds of modification, but one series of experiments were missing. To evaluate the efficacy of the peptides, we need to know whether the binding affinity was also modulated. The binding affinity could be done by the biacore binding assay by using the chemically synthesized peptides and purified recombinant proteins. In this way, we could also get some idea on whether modification changes the characteristics of the peptides.

For the future directions, first the 5 positive candidates for gephyrin should be evaluated by the biacore assay and the one with best binding affinity should be further tested for its knockdown efficacy and potential toxicity. Then the peptide was modified using the strategies I illustrated in the thesis to reduce the cytotoxicity while keeping the binding affinity. After the peptides were chosen and tested in vitro, both the modified peptides for gephyrin and PSD95 should be tested in vivo, for example in ASD mouse models or epilepsy to test their influence on the excitatory and inhibitory synapses. Since gephyrin was only responsible for certain subtype of $GABA_ARs$, the model for gephyrin could focus on the glycinergic inhibitory synapses in the spinal cord.

Taking all together, I figured out several ways from optimizing the treatment protocol to modifying the intrinsic properties of peptide to reduce the cytotoxicity. High dose short time treatment, low dose multiple treatment, and addition of linkers could all reduce the cytotoxicity significantly, but their influence on knockdown effect may limited their use. And the most promising way till now was to use the short CMA targeting motif. Our findings could enhance the potential of peptides as a therapeutic drug.

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