MOLECULAR AND FUNCTIONAL CHARACTERIZATIONS OF PROTEIN-PROTEIN INTERACTIONS IN CENTRAL NERVOUS SYSTEM

By

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A thesis submitted in conformity with the requirements for the degree of Doctoral of Philosophy
Institute of Medical Science
University of Toronto

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2011

Abstract

Many pathological processes are associated with excessive neurotransmitter release that leads to the over-stimulation of post-synaptic neurotransmitter receptors. Examples include excessive activation of glutamate receptors in ischemic stroke and hyper-dopaminergic state in schizophrenia and drug addiction. Thus, it would seem that simply antagonizing the involved receptors should be able to correct the pathological condition. In some instances, this strategy has been somewhat effective, such as with the use of dopamine D2 receptor antagonists as antipsychotics in the treatment of positive symptoms of schizophrenia despite severe side effects. However, clinical application of drugs antagonizing glutamate receptor in the treatment of stoke, although attracting intensive research effort, has been restricted by serious side effects caused by suppressing postsynaptic responses that are needed for normal brain function. As a consequence, it is important to develop novel therapeutics aiming at specific targets with minimized side effects. Numerous studies have suggested that the pathophysiology of neuropsychiatric disorders, drug addictions and stroke involves multiple neurotransmitter receptor systems such as the dopamine and glutamate systems. The activation or inhibition of one receptor can have cross-functional effect that will be better understood by investigating the functional and structural relationship between receptor systems. Thus, the present study has focused on characterizing receptor-receptor interactions associated with dopamine receptors and glutamate receptors, and to elucidate the physiological and pathological consequence of altered receptor interactions in schizophrenia, depression and ischemic stroke.
Acknowledgments

I would like to thank a number of people who have helped me throughout my Ph.D. program, but most importantly my supervisor, Dr. Fang Liu, for her support and guidance, and without whom none of this would be possible. Other people I want to thank are:

Dr. Min Zhuo and Dr. Albert Wong: Program Advisory Committee members who helped me interpret results, design experiments and consider alternatives to my hypotheses.

Dr. Lin Pei and Dr. Shupeng Li: Co-authors who provided helpful discussions and scientific collaborations.

Mr. Brian Vukusic: Best lab technician EVER.

Dr. Frank. J. Lee, Dr. Shengwei Zou, Dr. Hongyu Zhang, Dr. Qiang Nai, Dr. Heping Zhang, Dr. Anna Moszczenska, Dr. Sheng Chen, Dr. Dongxu Zhai, Dr. Ridong Wang and Mr. Terence Lai: Past and present members of the Liu Lab for making our lab a fun studying and working environment.

Mrs. Lori Dixon: Manager of CAMH animal committee who provided sincere help with animal studies.

My mom, dad and other family members: Constant encouragement helping me along the way.

Formal Acknowledgements:

Figures 2-1 to 2-8 are taken from the following manuscript:


Figures 3-1 to 3-9 and Table 3-1 to 3-2 are taken from the following manuscript:

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Chapter 1

1. Introduction

What are the main tasks of the central nervous system (CNS)? We have been seeking the answer to this question for hundreds of years but have yet been able to answer it completely. In general, human brain consists of three main functional components: the sensory system, the motor system and the homeostatic and higher brain functions. With the cooperation of these systems, our brain selects sorts and processes the overwhelming information from our bodies and environment, while guiding our behavioral and mental responses to our own body, other human beings and surroundings in according to the interpretation.

The basic element of CNS is the neurons, which are connected by axons to conduct cellular signals fast and precisely. In CNS, billions of neurons form highly organized networks including molecular (intracellular) and neuronal cellular networks for information processing and communication. During development, individual neurons make highly specific synaptic connections with others. On average, a neuron makes about 1,000 synaptic connections and receives many more. Therefore, there are more than one hundred trillions synaptic connections in a human brain.

The synapse, where the axon of one neuron connects to a dendrite of another, permits a neuron passes signals to another cell. This word comes from the Greek "syn-" ("together") and "haptein" ("to clasp"). The synapse consists of three elements: the pre-synaptic terminal, the synaptic cleft and the post-synaptic terminal. Generally speaking, the pre-synaptic terminal is located on the axon of the signal-passing neuron, and the post-synaptic terminal is with the membrane of the dendrite of the target neuron. However, some pre-synaptic sites are also found to be located on a dendrite or soma [1]. Based on the apposition, synapses are categorized into
two major types: electrical and chemical (Figure 1-1). In an electrical synapse, the pre- and post-synaptic cell membranes are connected by gap-junction channels that allow electrical current to flow from the presynaptic neuron into the post-synaptic neuron, and hereby alter the membrane potential in the post-synaptic cell. These gap-junction channels are permeable to small molecules and some second messengers (Figure 1-1, upper panel). Due to this direct mechanism, electrical transmission is the more rapid form of signaling between neurons. Groups of cells with electrical synapses can fire together when their collective threshold is reached. Because of the speed and the synchrony, electrical synapses are more suitable for fast and stereotyped behaviors.

In a chemical synapse, the synaptic cleft is larger and there are no bridging channels. The change in the membrane potential in the pre-synaptic neuron leads to the release of neurotransmitters that diffuse across the synaptic cleft and bind to receptors located on the post-synaptic cell membrane, thus initiating downstream molecular signaling pathways (Figure 1-1, lower panel). It is actually the receptor, rather than the neurotransmitter, that determines whether the synaptic response is excitatory or inhibitory. The same transmitter can produce different actions in different subtypes of receptors. For example, dopamine is a catecholamine neurotransmitter wildly expressed in the brain. By binding to a variety of dopamine receptors (D1-D5 receptors), dopamine can exert many different functions in the brain, including regulating neuro-endocrine functions, locomotor activity, cognition and emotion. Another example is glutamate, which is the principal excitatory neurotransmitter in the brain. By activating several major types of glutamate receptors such as NMDA, AMPA, kainate (ionotropic) and metabotropic glutamate receptors, glutamate plays an important role in numerous functions, including neuronal circuit development, learning and memory [2]. According to how the receptor and effector functions are coupled, chemical transmissions can be
Figure 1-1. Electrical and chemical synapses differ fundamentally in their transmission mechanisms.

**Upper panel:** At electrical synapses, gap junctions between pre- and postsynaptic membranes permit current to flow passively through intercellular channels. **Lower panel:** At chemical synapses, the secretion of neurotransmitters that open or close postsynaptic ion channels after binding to receptors.
divided into two different types: direct and indirect. Directly-gated chemical transmission is mediated by receptors that contain ion channel structure (e.g. ionotropic receptors), while the recognition of the transmitter and the activation of effectors are carried out by separate molecules in the indirectly-gated chemical transmission (e.g. metabotropic receptors).

Although even the fastest chemical synaptic mechanisms are slower than electrical synaptic transmission, chemical synaptic transmission has the advantage that a single action potential at the presynaptic site releases thousands of neurotransmitters into the synaptic cleft, allowing signals to be amplified from one neuron to another. While in return, several levels of the intracellular cascade feedback circuits can affect previous steps. Moreover, these parallel transmission lines can reciprocally interact at multiple levels, forming a complex network of signals starting from the membrane and going back to it. Numerous evidences indicate that interactions among transmission lines can take place at the level of the cell membrane via interactions among macromolecules (integral or associated to the cell membrane, i.e. receptors) involved in signal recognition and transduction. The present study will focus on this last subject, i.e., on the interactions between receptors for chemical signals at the level of the neuronal membrane ("receptor-receptor interaction").

1.1 Historical overview of the receptor-receptor interaction

1.1.1 Emergence of the receptor-receptor interaction

The classical view of cross-talk between receptors is a cluster of linearly organized events with divergence at the second messenger level in the cytoplasm (Figure 1-2). According to this classical view, the process starts to be divergent at the level of the second messenger. As shown in the upper panel in Figure 1-3, the interaction between receptors does not take place directly (the physical interaction between proteins or with the help of adapters or scaffold
proteins). The cross-talk can be mediated by two ways: 1) the activation of the first receptor induces the changes in membrane potential (ionotropic receptor) [3], and 2) the binding of ligand to the first receptor causes phosphorylation or de-phosphorylation by activating the intracellular cascade (metabotropic receptor) [4]. The change in the membrane potential or the altered phosphorylation state would then initiate a conformational change in the second receptor leading to altered ligand recognition and the downstream signaling pathway of the second receptor. However, this classical view was weakened by the discovery of the negative cooperativity in beta adrenergic receptors, which could be explained on the basis of the existence of homo-dimers leading to site-site interactions [5].

Since then, the concept of receptor-receptor interaction (RRI) has been introduced for three decades (Figure 1-3, lower panel). In 1980/81, Agnati and Fuxe discovered that substance P, which is a co-mediator in the bulbospinal 5-HT neurons, could change the binding characteristics of \( ^3\)H-5-HT binding sites in spinal cord membranes, and that cholecystokinin-8 modulated the characteristic of \( ^3\)H-spiperone binding sites-linked to dopamine (DA) D2 receptors in striatal membranes [6, 7]. This is the first experimental observation for the existence of direct physical intramembrane RRI, which can occur between different types of receptors. In the Wenner-Gren Centre symposium held in Stockholm in 1986, this discovery was then proposed to be widened to take place among different classes of macromolecules, such as receptors, ion channels and ion pumps [8].

In 1982, the Agnati and Fuxe teams proposed the existence of assemblies of multiple receptors of various types in the plasma membrane and named it as receptor mosaic (RM) for the proposed assembly, as a molecular basis for the engram [9, 10]. RM may be defined as: an assemblage of more than two receptors, which binds and decodes signals (transmitters, allosteric
Figure 1-2. Schematic illustration of classical view of cross-talk between receptors in a cluster of linearly organized events with divergence at the second messenger level in the cytoplasm.
Figure 1-3. Schematic illustration of classical and novel views of receptor-receptor interactions. **Upper panel:** indirect interactions involve changes in membrane potential or changes in phosphorylation/dephosphorylation of receptors. **Lower panel:** direct receptor-receptor interactions.
modulators, etc.) to produce an integrated input, via direct allosteric receptor-receptor interactions, to one or multiple intracellular cascades. The term RM gives a better vision of the integrative actions and the role of the spatial organization (topology) for the structure of the molecular fingerprint, giving rise to the intramembrane receptor-receptor interactions.

Following the above two steps, the third one was the proposal that the existence of the intramembrane receptor-receptor interactions made possible the circuit miniaturization with molecular networks formed in the surface membrane, suggesting that receptor-receptor interactions could allow the integration of synaptic (WT) and extrasynaptic (VT) signals [11-14]. According to this idea, cell signaling becomes a branched process beginning at the plasma membrane where the ligand recognition and signaling capacity of the receptors can be directly modified within a RM.

1.1.2 Cooperativity in intramembrane receptor-receptor interactions.

What are the different effects of ligands on receptor mosaics and the downstream signaling pathway? This major question has been discussed in many studies, but still remains unanswered. One possible biochemical explanation might be cooperativity, which takes place when a multimeric receptor binds to more than one molecule of the same transmitter. Cooperativity means that the binding of a ligand induces a sequential change of subunit conformation. The conformation change caused by the first ligand is transmitted intermolecularly to neighboring subunits in such a way that it might make subsequent ligands bind more easily (positive cooperativity), less easily or have no effect (negative cooperativity). Positive cooperativity is a mechanism to sharpen the responsiveness of a receptor system to a change of its ligand in a narrow range of concentrations. Negative cooperativity is a mechanism to dampen the responsiveness of a receptor system to a change of its ligand in a broad range of
concentrations to avoid overactivation of the receptor system. One of the most studied models of cooperativity is the tetrameric haemoglobin, in which both a concerted (all-or-noting) and a sequential (mixed conformational states) mechanism may exist [15].

Based on the above concept of cooperative, receptor mosaics may be divided into three main types: RM-type1 (RM1), RM-type 2 (RM2) and RM of a mixed type (RMm) [16].

- **RM1** consists of the same type of receptor (homo-oligomers, such as D2 dimers [17-20]) or of different subtypes of the same receptor (hetero-oligomers, such as D1-D2 [21-23] and D2-D3 [24-26]).
- **RM2** consists of different types of receptors (hetero-oligomers, such as GABA A-D5 [27] and NMDA-D1 [28]). The same type or subtype of receptors can be involved, but not in contact.
- **RMm**, as its name, consists of the mixture of the RM2 with the “RM1 Island”.

Among these three types of RM, RM1 can develop cooperativity because it is a receptor complex that binds one specific ligand. RM2 therefore cannot develop cooperativity because the complex binds different ligands. In this case, a broader concept of allosteric interaction can be employed, in which the binding of the first ligand makes the binding of the subsequent different ligand easier or more difficult. In RMm, small RM1 islands can show cooperativity and play an important role in affecting the function of the entire RMm.

These RRIs will greatly determine the various conformation states of these receptors and their operation will be determined by the composition, topography and order of receptor activation in the RMs [16, 29].
1.1.3 Horizontal molecular network and vertical molecular network

Because of the importance of RMs in the signal integration in CNS, their locations and the organization network where they belong to have to be carefully studied. Based on the location of RMs-related complex tree of signaling molecules in the cell compartment, the molecular networks can be divided into two types: horizontal molecular network (HMN) and vertical molecular network (VMN) [29].

- HMN is built up by membrane-associated or membrane-integral proteins, which are located within a membrane micro-domain or associated with the plasma membrane micro-domain. These molecules are physically interacted or communicated via a signal released and diffused within the membrane.

- VMN is the molecular network that stays inside of the cytoplasm, and often forwards the signals towards the nucleus.

As shown in Figure 1-4, HMNs are formed by different classes of molecules, and some of them are targets for other cytoplasmic and extracellular molecules. These molecules modulate receptor trafficking/desensitization and regulate receptor functions. HMNs are two-sided input/output networks, which can receive signals from both the extracellular and intracellular compartment of the cell. Due to this special property, HMNs can give responses affecting both the extracellular and the intracellular environment to maintain cell homeostasis.

Although multiple VMNs are potentially connected to HMNs, there are some molecules help choosing which VMN to activate when inputs reach HMNs. These molecules also give off their own signals to the intracellular biochemical machinery.
Figure 1-4. Schematic illustration of the horizontal molecular network and the vertical molecular network.
In the view of the concept of RMs, the RM1 acts as a crucial branch point in the membrane, where it interacts not only with membrane associated proteins to form the HMNs but also with proteins in the extra-cellular matrix and in the cytoplasm forming the so called VMNs [30]. Therefore, it will be important to understand the mechanisms underling the following questions: 1) how the receptors are organized within the RM to form HMNs; and 2) how they direct signals to various VMNs.

1.2 Biochemical implications

There are four major aspects of the biochemical implications of receptor-receptor interactions:

1.2.1 Altered receptor activity and G protein coupling.

Starting from the very first step, RRI are formed to be able to regulate the receptor activity and the G protein coupling in GPCRs, which result in the activation or inhibition of multiple molecules in the downstream signaling pathways. The D1-NMDA heteromer is an example of how RRI will modulate the receptor activation. In this case, electrophysiological recordings revealed that NMDA currents were significantly reduced after D1 receptor activation by agonist SKF81297. Furthermore, this reduction in NMDA currents mediated by the D1 receptor agonist was almost completely abolished by the interruption of the D1-NMDA interaction. In addition, these studies were performed in the presence of protein kinase A and C inhibitors suggesting that these effects on NMDA currents can be attributed to the physical interaction between D1 and NMDA receptors [28]. As for how the RRI regulate the G protein coupling, the D1-D2 heteromer is an example of how the formation of a RRI will modulate the coupling G protein. George et al found that the co-activation of D1 and D2 receptors lead the
D1-D2 heteromer couple to rapid activation of Gq/11 in the striatum, instead of Gs and Gi/o, respectively. Rather than going through the adenylate cyclase (AC) and cyclic AMP (cAMP) pathway when D1 or D2 receptors are stimulated alone, the D1-D2 heteromer activates the phospholipase C (PLC) and lead to intracellular calcium release [22, 23]. The increased level of calcium and the downstream calmodulin-dependent protein kinase IIα (CAMKIIα) contribute to the alteration of synaptic plasticity [31].

Due to the intense relationship between the interacted receptors, it should be noted that, in the pathological condition, any slight alteration in one of the above processes can cause abnormalities in the sensitivities of several receptors and in the activation or inhibition of the downstream signaling molecules.

1.2.2 Altered pharmacological properties

The RRI may modulate the pharmacological properties of the involved receptors like the GABA B receptor [32]. It has been reported that the class C GABA B receptor is a heteromer formed by GABA B1 and GABA B2 receptors. The GABA B1 receptor binds to the ligand and passes the signal from the flytrap domain to the TM domain of the GABA B2 receptor via allosteric mechanisms. Another example is the A1R/P2Y1R heteromer, in which the pharmacology of the A1R binding pockets shows P2Y agonist-like property [33, 34]. The D1-D2 heteromer discussed above also displays a unique pharmacological property [23, 35]. It was found that the D1 agonist SKF 83959 was a selective agonist for this RM acting as a full agonist of D1 receptor and a partial agonist of D2 receptor. The application of SKF 83959 to the D1-D2 heteromer leads to the activation of PLC and the following intracellular calcium release. However, this SKF 83959 exerts no effect on the AC pathways linked with D1 or D2 receptors suggesting that SKF 83959 may be a unique agonist for the D1-D2 RM. It has also been reported
that the desensitization of this Gq/11-mediated calcium signal was demonstrated by pretreatment with dopamine or with the D1-selective agonist SKF-81297 or the D2-selective agonist quinpirole. Recent studies have reported that the calcium signaling-mediated increase in CaMKIIα is required for the cocaine addiction [36], while the link is diminished in schizophrenia patients [37].

1.2.3 Formation of novel interactions

The formation of RM could lead to alterations of the structure and the topology of receptors involved. These conformational changes may result in the formation of novel bindings with other receptors, such as other GPCRs or ligand-gated ion channels. In addition, the novel formation could also include the interaction with the membrane-associated proteins, including scaffolding proteins, chaperones and adaptors. Altogether here comes the HMN, in which the RM acts as a crucial node linking every component in the HMN and initiates the downstream signaling pathways in the VMN towards the nucleus. The dysfunction of this crucial node could lead to the formation of the abnormal HMN, and thus cause either the inactive or overactive cellular response in the VMN.

1.2.4 Altered receptor trafficking and internalization

1.2.4.1 Trafficking

Let’s take GPCR trafficking as an example. Following synthesis, GPCRs initially reside in the endoplasmic reticulum (ER), where they undergo processing and folding guided by chaperone and quality-control proteins [38]. Within the ER, many GPCRs likely form homo- or heterodimeric structures. Following the departure from ER, GPCRs transit through the Golgi apparatus, where they may undergo additional modifications such as oligosaccharide processing.
On the distal edge of the Golgi, GPCRs are packaged into exocytotic transport vesicles and enter the endosomal system, where they are subsequently targeted to the plasma membrane [39]. One of the first discoveries that GPCR dimerization is important for receptor processing and targeting to the plasma membrane is done on the GABA_B receptor in 1998 [40-42]. It was found that the formation of a functional GABA_B receptor requires the co-expression of the two isoforms of the GABA_B receptors, GABA_B1 and GABA_B2 [43, 44]. Without the expression of GABA_B2, the GABA_B1 stays as an immature protein and retains within the cytoplasm [45]. On the other hand, GABA_B2 can be transported to the plasma membrane when expressed alone, but is unable to bind GABA. During the folding process in the ER for this heteromer, GABA_B2 acts as a chaperone which is crucial for the proper folding of GABA_B1 and cell surface transport of the RM [45]. The interaction between the carboxyl tails of the two isoforms leads to the masking of the ER-retention signal, which therefore allows ER transport and cell surface targeting of the heteromer. Another example is the β2-AR that undergoes constitutive homodimerization in the ER. It has been reported that the expression of mutant β2-ARs constructed to either lack an ER-export motif or contain a heterologous ER-retention signal leads to entrapment of wild-type β2-AR in the ER, likely because of receptor dimerization [46]. Moreover, the addition of a peptide encoding the putative glycophorin-like dimerization motif in the sixth-transmembrane domain of the β2-AR inhibits both the receptor dimerization and the transit to the plasma membrane [47]. Similar results in which the D2 receptor acts as dominant negatives for the cell surface expression of its wild-type receptor suggest that homo- and heteroreceptor oligomerization occurring at an early time point during receptor biosynthesis and maturation in the ER and Golgi apparatus potentially has an important role in the quality control of newly synthesized receptors [48].
1.2.4.2 Internalization

The D1 and adenosine A1 receptor (A1R) heteromer has been found both in cell lines and in striatum [49-53]. It is worth noting that pretreatment with the A1R agonist causes co-clustering (coaggregation) of A1R and D1R, which is blocked by combined pretreatment with the D1R and A1R agonists in both fibroblast cells and in cortical neurons in culture. Combined pretreatment with D1R and A1R agonists, but not with either one alone, substantially reduced the D1R agonist-induced accumulation of cAMP [52], which means that desensitization is only possible after co-activation. The mechanism for the D1R desensitization may be that co-activation of the D1R and A1R develops the well documented antagonistic A1/D1 receptor-receptor interactions found in the neuronal networks of the brain. As a result, an A1R agonist after a 3-hour exposure produces enduring conformational changes of the D1 receptor via the antagonistic A1/D1 interactions leading to reduced D1 signaling. D1 phosphorylation and the binding of β-arrestins to the D1 receptor may also contribute to this phenomenon. In contrast, the D1-D2 heteromer that we have discussed above has no antagonistic receptor-receptor interaction; instead the co-activation leads to co-internalization [54, 55]. Because of the formation of D1/NMDA receptor-receptor interaction mediated by the carboxyl tails of both receptors, the activation of NMDA receptors increases the number of D1 receptors on the plasma membrane surface and enhances D1 receptor-mediated cAMP accumulation via a SNARE-dependent mechanism. Furthermore, over-expression of mini-genes encoding either NMDA or D1 carboxyl tail fragments disrupts the D1-NMDA direct protein-protein interaction and abolishes NMDA-induced changes in both D1 cell surface expression and D1-mediated cAMP accumulation [56]. Scott et al. also found that D1 receptors that diffuse in the surface membrane of spines can be trapped by activated NMDA receptor contributing to the formation of the D1/NMDA RMs [57].
Unlike A1-D1 heteromers, in D2 co-transfected neuroblastoma cells, co-activation of A2A-D2 heteromer leads to co-aggregation, co-internalization and co-desensitization of A2A and D2 receptors [58, 59]. In contrast to the effects of the agonist treatment, a three-hour treatment with the D2-like antagonist raclopride increased both A2A and D2 immunoreactivity in CHO cells, indicating that the D2 antagonist stabilizes the D2 receptor and thereby reduces the internalization of both of the A2A and D2 receptors. Therefore, it seems likely that the trafficking properties among the various types of heteromers may vary considerably and no general rules for their trafficking behavior can be introduced, at least at this stage.

1.3 Pathological implications and New drug development

In both animal and human studies, there has been considerable evidence demonstrating the important role of receptor-receptor interactions in neurological disorders. Therefore, regulation of receptor-receptor interactions may become a potential target for the new drug development.

1.3.1 Depression

Major depression is multi-dimensional in its symptom presentation across the patient population, with a complex etiology and pathophysiology [60]. The core symptoms include depressed mood, fatigue, and anhedonia, as well as impaired attention, low self-esteem, guilt, pessimism, weight change, and suicidal ideation [61-63]. There has been a large body of independent evidence suggesting that 5-HT function is abnormal in depression. Among 5-HT receptor subtypes, 5-HT-1A receptors have long been implicated in the pathogenesis and in the treatment of anxiety and depressive disorders. In 1988, galanin was shown to reduce the affinity of 5-HT-1A receptors in the ventral limbic cortex, which is the first evidence for the existence of antagonistic intramembrane GalR/5HT-1A RM [64]. Three years later, Fuxe and colleagues
reported the reciprocal interaction showing that 5-HT-1A receptor activation leads to an increase in the affinity of GalR in various regions of the tel- and diencephalons [65]. The mechanism underlying this reciprocal regulation may be the inhibitory feedback to reduce the overactivation of 5-HT-1A signaling via the GalR/5-HT-1A RM. Consistent with these previous studies, it has also been found that chronic treatment with imipramine could increase its affinity for the galanine receptors binding sites in the tel- and diencephalon, which may be caused by the increased activation of 5-HT-1A receptor. In addition, the overactivation of 5-HT-1A receptor is due to the increased extracellular levels of 5-HT caused by the blockade of the 5-HT transporter by imipramine. Furthermore, galanin receptor antagonists may also produce antidepressant effects by blocking galanin receptors in the dorsal raphe, which inhibit the 5-HT releasing activity and firing of the ascending 5-HT pathways to the tel- and diencephalon. Therefore, the present evidence would strongly favor the development of the antidepressant drugs with galanin receptor antagonist properties via GalR/5-HT-1A receptor-receptor interaction to regulate 5-HT signaling in depression.

1.3.2 Schizophrenia

Schizophrenia is a severe psychiatric disorder, which affects 1% of the population worldwide. Many factors have been found to be associated with an increased risk to develop schizophrenia [66]. In general, schizophrenia is considered a complex disease with multiple factors contributing to the pathogenesis. In CNS, multiple neurotransmitter systems and functional networks have been reported to be affected in schizophrenia patients. Whether these alterations are causative for the development of disease, or rather are consequences of the treatment is still not completely understood. Chlorpromazine, the first drug found to alleviate the symptoms of schizophrenia, was introduced in 1952 [67], followed by other typical antipsychotic
drugs, such as haloperidol [68, 69]. The target of all these antipsychotic drugs turns out to be dopamine D2 receptor, from which arises the most influential and longest enduring dopamine hypothesis of schizophrenia. In brief, this theory postulates that excess dopaminergic neurotransmission especially in mesolimbic and striatal regions leads to positive symptoms, while dopaminergic deficits in prefrontal regions are responsible for the negative symptoms. Over the past five decades, blockade of D2 receptors still plays key role in mediating the antipsychotic drugs [70] and recent studies have demonstrated that A2A agonists may be novel antipsychotic drugs by antagonizing the D2 receptor signaling via an A2A/D2 intramembrane receptor-receptor interaction in the ventral striato-pallidal GABA system [71-73]. The A2A agonist, CGS21680, was shown to have an atypical antipsychotic profile by reducing the amphetamine (AMPH) and PCP-induced locomotor activity in doses failing to cause catalepsy. Furthermore, the injection of the A2A agonist into the nucleus accumbens (NAC) reversed the inhibition of prepulse inhibition by apomorphine [74] and combined treatment with subthreshold doses of a D2 antagonist and an A2A agonist led to an activation of the ventral striato-pallidal GABA pathway [72]. Moreover, the increase in fos-like immunoreactivity in the NAC after treatment with antipsychotic drugs like clozapine and haloperidol was counteracted by treatment with an A2A antagonist [75]. It is of substantial interest that the A2A agonist demonstrates antipsychotic-like activity in Cebus appella monkeys without production of extrapyramidal side effects, underlining the development of novel A2A agonists as a strategy for treatment of schizophrenia [76]. In addition to the A2A/D2 receptor-receptor interaction, A2A/D3 receptor heteromers with antagonistic A2A/D3 receptor interactions have also been demonstrated in co-transfected CHO cell lines [77]. Moreover, recent studies suggest that synergistic interactions between A2A and mGluR5 receptors based on the existence of A2A/mGluR5 heteromeric complexes in postulated extrasynaptic mGluR5/A2A/D2 RM5 of the ventral striato-pallidal
GABA neurons play a major role in increasing activity of this pathway and removing it from D2-mediated inhibition [78]. Therefore, it is reasonable to suggest that drugs with combined A2A agonist and mGluR5 agonist properties may have antipsychotic properties by restoring the drive in the ventral striato-pallidal GABA pathway through counteraction of D2 signaling.

Although the dopamine hypothesis remains as the most widely considered theory, the glutamate hypofunction hypothesis of schizophrenia has been put forward as a supplementary and unifying biochemical model of the disease [79, 80]. In this concept, a hypofunction of glutamate in cortico-striatal projections leads to an opening effect in the thalamo-cortical loop resulting in an exaggerated sensory flooding and thereby psychotic symptoms and the well-known dopamine concentration changes. The glutamate hypothesis arises from the observation that drugs such as phencyclidine (PCP) and ketamine, both of which are non-competitive antagonists of the NMDA receptor, reliably and immediately lead to effects that mimic those seen in schizophrenia including hallucinations, delusions, thought disorder and, most notably, negative symptoms [81]. Despite the growing evidence for glutamatergic dysregulation in schizophrenia, dopamine D2 receptor blockade still appears to be the only absolute requirement for the antipsychotic effect of all currently available antipsychotic drugs [70]. If glutamate dysregulation is important, it may be predicted that glutamate release must either be extensively modulated by the dopamine system or, alternatively, must lead to downstream effects at the dopamine system that subsequently give rise to psychotic symptoms. Besides D2 receptor, dopamine D1 receptor has also been suggested as one of the potential antipsychotic agents recently, particularly with respect to cognitive deficits in schizophrenia. D1 receptors are the primary postsynaptic dopamine receptor in the cortex, and it has been hypothesized that the cognitive dysfunction in schizophrenia may be related to the reduced dopamine neurotransmission in the frontal cortex. In animal studies, there is evidence that chronic PCP and
MK-801 administration both lead to a reduction in frontal dopamine release [82] and an increase in D1 receptor binding in vivo, quantified using PET [83]. In schizophrenia patients, there are also growing evidence showing reduced frontal dopamine neurotransmission and upregulated frontal D1 receptors [84]. Recent studies demonstrate that the reciprocal regulation exists between D1 and NMDA receptors via direct D1/NMDA receptor-receptor interaction [28, 56, 85]. Kandel and colleagues recently demonstrated that D1 receptor activation is necessary for maintaining long-term potential (LTP) and depression at glutamatergic synapses in the prefrontal cortex [86]. Liu team also discovered that activation of D1 receptor promotes NMDA receptor-dependent LTP and working memory in hippocampus [85]. Thus, understanding the molecular mechanisms by which D1 and NMDA receptors functionally interact may provide insight toward elucidating the molecular neurobiological mechanisms involved in schizophrenia.

1.3.3 Cell Death

Patients with chronic neuropsychiatric diseases, such as depression and schizophrenia discussed above, may be at particular risk of neuronal loss through cell death. Numerous in vivo imaging studies report selective functional and structural changes in limbic structures such as the prefrontal cortex and hippocampus in patients with major depressive disorder [87-91]. Probably the most reproduced finding is a small (10–15%) but significant reduction in hippocampal volume as documented by in vivo magnetic resonance imaging (MRI) studies [92, 93]. Moreover, duration of the depressive episode is closely paralleled by volumetric changes, with longer periods of depression generally corresponding to smaller hippocampi [90, 91, 94]. Even though the possibility cannot be excluded that a smaller hippocampal volume might be a trait characteristic of depression [95], it should be noted that hippocampal shrinkage is not specific to
depression and has been reported in a number of other neuropsychiatric and neurological disorders including schizophrenia [96] and traumatic brain injury [97].

Although schizophrenia is generally considered a neuro-developmental disorder, evidence for progressive clinical deterioration and subtle neuro-structural changes following the onset of psychosis has led to the hypothesis that cell death may contribute to the pathophysiology of schizophrenia. MRI studies have demonstrated evidence of reduced cortical gray matter volume, both globally [98-100] and in sub-cortical regions including prefrontal [98, 99], temporal [98, 101], and parietal areas [101].

NMDA hypofunction, glutamate excitotoxicity and altered calcium signaling have all been implicated in the pathophysiology of depression [102-104] and schizophrenia [79, 105, 106]. High calcium levels, oxidative stress, and mitochondrial dysfunction can all lead to glutamate excitotoxicity and each can also promote apoptotic activities [107]. Even very brief NMDA receptor blockade during vulnerable intervals during rat development can greatly augment normal developmental neuronal apoptosis [108]. Previous studies have shown that NMDA antagonism can also produce long-term changes in Bcl-2 gene family expression including higher pro-apoptotic Bax and lower anti-apoptotic Bcl-XL as well as increase expression of the NMDA NR1 receptor subunit [109]. Furthermore, altered expression of the NMDA NR1 and NR2A receptor subunits can increase the apoptotic vulnerability of neurons [110]. Emerging data implicate changes in these NMDA subunits and others in the postmortem neuropathology of schizophrenia [111]. Of particular interest, altered editing of GluR2 mRNA of the AMPA receptor–as has been identified in schizophrenia cortex [112]–has been associated with significantly higher calcium influx. Such influx can lead to neuronal atrophy and apoptosis [113, 114]. Thus, while the implications of glutamatergic dysfunction in schizophrenia remain uncertain, it presents as a candidate mechanism that can
adversely impact neuroapoptotic processes. In particular, the susceptibility of cortical neurons to NMDA antagonists during neurodevelopment suggests a plausible scenario for a transient activation of apoptosis that could exert limited neurostructural effects early in the course of schizophrenia. Therefore, regulating NMDA and AMPA receptor function via receptor-receptor interactions, such as D1/NR1, D2/NR2B and D2/GluR2, may be a potential novel therapeutic strategy for the cell loss in neuropsychiatric diseases.

1.3.4 Stroke

Excessive extracellular exposure to glutamate is harmful to neurons and contributes to neurodegeneration in both chronic conditions as discussed above, and acute conditions, such as ischemic stroke. Loss of cerebral blood flow rapidly triggers energy deficits and neuronal depolarization that release large amounts of glutamate into extracellular space. Glutamate activates two major subfamilies of ligand-gated ion channels: NMDAR and AMPAR [115]. Overactivation of these two types of the glutamate receptors lead to a generalized ionic imbalance within neurons, especially of calcium. The calcium overload is then thought to induce a wide range of cell death executioners including ATPases, proteases, lipases and DNAses [116-119]. Consequently, stabilization of intracellular calcium has been a major objective in the search for a therapeutic method of minimizing the brain damage that follows stroke. However, neuroprotection via glutamate receptor blockade therapy has not proven effective in the clinical treatment of brain damage due to narrow therapeutic windows, poor pharmacokinetics and most importantly, the blockade of the signaling essential for normal excitatory neurotransmission and neuronal survival [120, 121]. Therefore, it is of great interest to regulate glutamate receptors function without completely blocking the receptor.
Dopamine is known to regulate the function of several proteins, including those important in ischemic stroke, such as glutamate receptors and ATPase. Underlying the complexity of dopamine–glutamate interactions is the co-localization of dopamine and glutamate receptors, including D1 and NMDA receptors, within several brain structures including caudate-putamen, nucleus accumbens, hippocampus and rat forebrain as determined through autoradiography and immunocytochemistry [122-132]. Traditionally, many of the effects of dopamine receptor activation are thought to be dependent on the PKA pathway that regulates the phosphorylation state of multiple sites on the dopamine- and cAMP-regulated phosphoprotein of 32 kDa (DARPP-32). For example, phosphorylation of Thr34 on DARPP-32 by PKA after D1 receptor activation inhibits protein phosphatase-1 and thereby permits sustained phosphorylation of serine and threonine sites on other proteins targeted by PKA [133]. Stimulation of D1 receptors leads to phosphorylation of Ser897 on the NR1 subunits of NMDA receptors [134, 135] by a mechanism dependent on DARPP-32 [136], which can increase receptor sensitivity to glutamate and increase calcium currents [137, 138]. However, in recent years, another paradigm has emerged that involves the direct interaction between D1 and NMDA receptors. The physical association between D1 and NMDA receptors is unique in that two different regions of the D1 C-terminus are able to couple specifically and physically with two different NMDA subunits. These two D1-NMDA interaction sites can modulate NMDA currents and affect NMDA-mediated excitotoxicity, respectively [28]. In addition to D1 receptor, activation of D2 receptor may also attenuate NMDA-mediated current via direct D2-NR2B interaction [139].

Similar to NMDA receptors, considerable evidence have shown that ischemia can cause the alteration of the expression pattern of AMPA receptor subunits in certain brain regions [140-142] and AMPA receptor antagonists exert protective effects against ischemia-induced neuronal death [143-145], suggesting that AMPA receptors also play an important role in post-ischemic
neurodegeneration. Previous studies showed that AMPA receptor phosphorylation/dephosphorylation, by cAMP-dependent protein kinase (PKA), protein kinase C (PKC), or calcium-calmodulin kinase (CaMK), can regulate synaptic glutamatergic activity [146-149]. Recent studies have shown that a variety of intracellular proteins directly bind to AMPA receptor subunit carboxyl tail (CT) regions and play an important role in receptor targeting, membrane expression, internalization, clustering, and in the modulation of receptor activity and activation [150-155]. More interestingly, activation of D2 receptor may inhibit AMPA receptor-mediated excitotoxicity via the D2-AMPA receptor complex. Zou et al. reported that agonist stimulation of D2 receptors promotes the formation of the direct protein-protein interaction between the third intracellular loop of the D2 receptor and the N-ethylmaleimide-sensitive factor (NSF) and uncouples the NSF interaction with the CT of the glutamate receptor GluR2 subunit of AMPA receptors. The enhanced coupling of D2-NSF and the diminution in the NSF-GluR2 interaction do not only result in a decrease of AMPA receptor membrane expression, but also facilitate the GluR2-p85 interaction and subsequent activation of PI-3K, which initiates anti-apoptotic pathways [156].

Ischemic stroke is a worldwide public health problem and one of the leading causes of death. More than three decades have passed, however, clinical applications of glutamate receptor antagonists for the treatment of ischemia stroke yet remains unsuccessful. Therefore, recent focus has shifted to studies characterizing receptor-receptor interactions associated with glutamate receptors, which may provide intriguing therapeutic strategies for treating stroke and neurodegeneration in the coming years.
1.4 R-R interactions in molecular medicines

As discussed above, the existence of classical and non-classical cooperative interactions in RRIs leads to the formation of novel interactions. Therefore, the pharmacological properties of the binding pockets of the different receptors building up the receptor complex can turn to be remarkably different versus their pharmacology in the respective homomers. This unique property of RRIs make it possible for the use of combined agonist or antagonist treatment to optimally increase or inhibit the signaling of one malfunctioning receptor in the complex. On the basis of the existence of μ–δ opioid receptor heterodimers with δ ligands enhancing the efficacy of μ agonists, bivalent compounds have inter alia been developed built up of a μ agonist linked to a δ antagonist that show special analgesic properties [157]. The distance between the two components modulated the appearance of opioid tolerance and dependence giving indications that the bivalent ligands in fact targeted the μ–δ opioid receptor heterodimers. Recent studies have also shown that the anti-Parkinsonian action of L-dopa treatment might, in part, be caused by the simultaneous chronic activation of A2A and D2 receptors [50]. The co-administration of A2A antagonists with L-dopa can reduced the doses of the combined drugs in the treatment of Parkinson’s disease, which reduces the appearance of side effects but maintaining therapeutic effects.

The formation of RRIs can produce novel interactions among distinct types of receptors, such as GPCRs bind with ligand-gated ion channels. These novel formations may appear or disappear in the brain membranes depending on the extrinsic and intrinsic cooperativity of the RRIs. Therefore, therapeutic agents targeting at facilitating or disrupting the formation of the novel ligand cognition-signal decoding systems may become a novel tool for the treatment of neurological disorders.
1.5 Summary

In conclusion, the discoveries of intramembrane receptor-receptor interactions that make the integration of signals at the level of the surface membrane possible have raised a novel principle in molecular medicine. By regulating the receptor trafficking, biochemical characteristics and the composition of the receptor complex, the receptor-receptor interactions open up new targets for treatment of receptor dysfunctions and open new doors for the discovery of more selective and highly effective drugs in neurological and mental disorders.

1.6 Research rational and objects

Many pathological processes are associated with excessive neurotransmitter release that leads to the over-stimulation of post-synaptic neurotransmitter receptors. Examples include excessive activation of glutamate receptors in ischemic stroke [158, 159] and hyper-dopaminergic state in schizophrenia [160, 161] and drug addiction [162]. Thus, it would seem that simply antagonizing the involved receptors should be able to correct the pathological condition. In some instances, this strategy has been somewhat effective, such as with the use of dopamine D2 receptor antagonists as antipsychotics in the treatment of positive symptoms of schizophrenia despite severe side effect. However, clinical application of drugs antagonizing glutamate receptor in the treatment of stoke, although attracting intensive research effort, has been restricted by serious side effects caused by suppressing postsynaptic responses that are needed for normal brain function. As a consequence, it is important to develop novel therapeutics aiming at specific targets with minimized side effects. Numerous studies have suggested that the pathophysiology of neuropsychiatric disorders, drug addictions and stroke involves multiple neurotransmitter receptor systems such as the dopamine and glutamate systems [163]. The activation or inhibition of one receptor can have cross-functional effect that will be better
understood by investigating the functional and structural relationship between receptor systems. Thus, the present study has focused on characterizing receptor-receptor interactions associated with dopamine receptors and glutamate receptors, and to elucidate the physiological and pathological consequence of altered receptor interactions.
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Chapter 2

Schizophrenia, Amphetamine-Induced Sensitized State and Acute Amphetamine Exposure All Show a Common Alteration: Increased Dopamine D2 Receptor Dimerization

Published previously as


Contributions of other authors:
I performed all experiments described in the paper with the following exceptions:

• Dr. Lin Pei helped prepare the acute rat striatal slices
• Dr. Paul Fletcher provided the AISS animal model
• Dr. Philip Seeman’s lab performed the [³H]-dopamine binding assay (Figure 2-5)
2.1 Introduction

Schizophrenia is a chronic mental illness, characterized by episodes of psychotic symptoms that usually emerge in early adulthood, last a lifetime, and destroy the mental and interpersonal faculties most valued in human society. The fact that all current antipsychotic drugs exert their effect through the blockade of D2R emphasizes the critical role of hyper-dopaminergic neurotransmission through D2R signaling in the pathophysiology of schizophrenia [1]. Yet, studies are conflicting about the role of D2R in the illness. While postmortem studies have demonstrated an increase in the overall number of D2R, this is confounded by the fact that this increase could be a compensatory response to D2R blockade rather than a diagnostic feature of schizophrenia [2]. On the other hand, PET imaging studies show unequivocal support for a change in D2R [3]. Several recent PET imaging studies show a well-replicated increase in dopamine release in patients with schizophrenia, and even during the prodromal phase [4]. Thus, it remains unclear what, if any, the direct role D2R play in the pathophysiology of schizophrenia.

Most previous studies have measured D2R using receptor radioactive ligand binding assay, PET imaging or Western blot analysis. These techniques provide a good index of overall binding sites, but do not provide any insight into molecular aspects of receptor functions, including their potential configurations with other receptors. Several GPCRs exist in homo- or hetero-dimers to allow multiple signal integrations [5]. A number of studies have focused on the role of dimerization in regulating the function of GPCRs [5]. Indeed, dimerization of GPCRs may alter pharmacological properties and signaling transduction, leading to significant effects on cellular physiology as well as disease pathologies. For example, agonist occupancy of one of the two ligand binding site of the β2-adrenoceptor dimers is sufficient to cause internalization of the dimers. However, both binding sites have to be occupied by the antagonist to prevent agonist-
mediated internalization [6]. Furthermore, receptor hetero-dimerization makes it possible to modulate one receptor using ligands targeting the other receptor [7-9]. These unique properties of receptor oligomerization have made them novel targets for the development of novel drugs [10].

Previous studies indicate that D2R are expressed as monomers and dimers in cell lines and in mammalian brain tissue [11-14]. Furthermore, D2R have also been reported as dimers in a variety of neurological disease such as Alzheimer’s, Parkinson’s and Huntington’s disease [15, 16]. As a result, interest has focused on the potential pathophysiological role of D2R dimerization in disease. Although pharmacological studies have suggested that D2R dimers and monomers have differential affinity for specific dopamine receptor ligands that may subsequently affect dopamine release [17, 18], the functional role of the D2R dimerization and the molecular mechanisms involved are not well understood.

Given the fact that all antipsychotics work on D2Rs and that D2Rs form dimers in-vitro and in-vivo, we hypothesized that schizophrenia, as well as preclinical models of schizophrenia, would demonstrate altered dimerization of D2Rs.
2.2 Results

2.2.1 Enhanced expression of D2R dimers in postmortem striatal sections from schizophrenia patients

As an initial step to investigate whether the expression pattern of D2R might be altered in schizophrenia, we carried out Western blot analysis with all 60 formalin-fixed paraffin-embedded human postmortem striatal sections from the Stanley Foundation, including 15 samples from each of four groups: control, bipolar disorder (BD), major depressive disorder (MDD) and schizophrenia (SZ). A polyclonal anti-D2 antibody that can specifically recognize both D2R dimers and monomers was used to examine the D2R expression in Western blot analysis (Figure 2-1). As shown in Figure 2-2 A-B, the expression level of D2R dimers exhibited a significant increase (277.7±33.6%) in the postmortem striatal sections of schizophrenia patients (n=15, p<0.001), whereas the expression of D2R monomers showed a substantial decrease (69.3±7.3%, Figure 2-2 A, C). These data suggest D2R dimerization may contribute to the pathophysiology of schizophrenia.

We are aware of the fact that most of the schizophrenia patient samples (13/15) we examined were exposed to antipsychotic treatment ante-mortem. Thus, to rule out the possibility that the observed enhancement of D2R dimerization in the human postmortem schizophrenia striatal sections is the consequence of antipsychotic treatment, we tested the expression of D2R dimerization in striatal brain tissue of rats chronically treated with haloperidol. As shown in Figure 2-3 A-B, haloperidol treatment failed to alter the degree of D2R dimerization. Furthermore, AMPH-induced D2R dimerization was not affected by the haloperidol treatment in rat striatal slices (Figure 2-3 C-D). Taken together, these data suggest that the observed increment of D2R dimers may not be caused by the antipsychotic treatment.
Figure 2-1. Specificity of D2R antibody.

A. Western blot analysis of dopamine receptor D2 dimer and monomer expression in both rat and human striatal extracts.
Figure 2-2. D2R dimers expression increased in postmortem striatal tissue of schizophrenia patients.

A. Representative Western blot analysis of the expression levels of D2R dimers and monomers in human postmortem striatal sections of control, bipolar, depression and schizophrenia groups. α-tubulin was used as a loading control.

B-C. Bar graphs summarizing the Western blot data of D2R dimers (B) and monomers (C). *, ***, Significantly different from control group ($p<0.05$, $p<0.001$; $n=15$). Data were analyzed by One-way ANOVA, followed by SNK post-hoc test.
Figure 2-3. The effect of antipsychotic treatment on D2R dimers expression.

**A-B.** Chronic haloperidol treatment caused no significant changes in the expression of D2R dimers and monomers in rat striatal tissue compared to control group (n=12). Data were analyzed by *t*-test.

**C-D.** Western blot analysis of the expression levels of D2R dimers (C) and monomers (D) in rat striatal slices treated with AMPH (30min, 10 μM), in the presence or absence of haloperidol (30min, 10 μM). *, **Significantly different from control group (*p*<0.05, **p***<0.01; n=3). Data were analyzed by One-way ANOVA, followed by SNK post-hoc test.
2.2.2 D2R dimerization is up-regulated in the striatal tissue of AISS rats

Due to the similarity between schizophrenia and amphetamine psychosis [26-28], AISS has been widely used as an animal model of schizophrenia. Thus, we investigated whether the expression pattern of D2R is also altered in the striatum of AISS rats. As shown in Figure 2-4 A-B, the expression of D2R dimer was significantly increased (415.3 ± 56.6%) in the AISS group compared to controls; the D2R monomer was significantly reduced in the AISS group compared to the control group (19.6 ± 5.0%, Figure 2-4 A, C). These data suggest that chronic amphetamine exposure, which leads to a sensitized state and models many aspects of the clinical illness, also displays the same alteration that is seen in the postmortem striatal sections of schizophrenia patients.

2.2.3 D2R dimerization may be associated with D2high receptors

Previous studies have shown that in many animal models for psychosis, including the AISS, the proportion of D2R in the high-affinity state is elevated [29]. However, the molecular basis that renders D2R super-sensitive to dopamine remains unclear. As both D2R dimers and D2high receptors are enhanced in the AISS animal model, we speculate that D2R dimerization may correlate with the D2R high-affinity state. We tested our hypothesis in rat striatal slices treated with amphetamine. Thus, rat striatal slices were treated with amphetamine and divided randomly into two groups: one for D2R dimer measurement and the other for D2high receptors measurement. As shown in Figure 2-5 A-B, acute amphetamine treatment significantly enhanced the expression of D2R dimers (295.0 ± 75.4%), while the D2R monomer expression was significantly decreased (39.9 ± 19.5%). Similarly, acute treatment with amphetamine increased the proportion of high-affinity D2R from 17 ± 1.4% to 38.8 ± 3.9% (Figure 2-5 C), examples of which are shown in Figure 2-5 D. These data suggest that D2R dimers may be associated with
Figure 2-4. Amphetamine induces D2R dimerization in AISS rat striatum.

**A.** Western blot analysis of the expression levels of D2R dimers and monomers in striatal extracts of control and AISS rats. α-tubulin was used as a loading control.

**B-C.** Bar graphs summarizing the western blot data. *, **Significantly different from control group ($p < 0.05, p < 0.01; n=5$). Data were analyzed by $t$-test.
**Figure 2-5. Amphetamine facilitates D2R dimerization and the expression of high affinity D2 receptors (D2high) in rat striatal brain slices.**

*A-B.* Western blot analysis of the expression levels of D2R dimers (A) and monomers (B) in rat striatal slices treated with or without AMPH (30min, 10 μM). *Significantly different from control group (p<0.05; n=3). Data were analyzed by t-test.

*C.* Bar graph summarizing the binding of [³H]domperidone in rat striatal slices treated with or without AMPH (30min, 10 μM). **Significantly different from control group (p<0.001; n=7 in control group and n=6 in AMPH group).

*D.* Graphs representing three samples out of six experiments. The competition between dopamine and [³H]domperidone showed a biphasic pattern. In control striata, low concentrations of dopamine (generally between 10 and 5,000 nM) inhibited the binding of [³H]domperidone by an average of 17 ± 1.4%. Higher concentrations of dopamine further reduced the binding of [³H]domperidone in a distinctly separate phase. There was a clear plateau between the high-affinity phase (i.e., at low concentrations of dopamine) and the low-affinity phase (i.e., at high concentrations of dopamine). The treatment with amphetamine increased the proportion of high-affinity D2 receptors to 38.8% ± 3.9%
the super-sensitivity of D2R to dopamine.

2.2.4 Amphetamine facilitates D2R dimerization in primary culture of rat striatal neurons.

In order to identify the factors involving in the process of amphetamine-induced D2R dimerization, we then tested the amphetamine effect on D2R dimerization in a simplified experimental condition. Primary culture of rat striatal neurons was treated with 10μM amphetamine for 30 min at 37 ºC. Consistent with the result from AISS rats, acute amphetamine treatment facilitated D2R dimerization (537.5 ± 37.5%, Figure 2-6 A), whereas the expression of D2R monomers was significantly decreased (14.9 ± 1.4%) in amphetamine-treated groups (Figure 2-6 B). Since amphetamine is able to enhance synaptic dopamine, we then tested whether the observed amphetamine-induced up-regulation of D2R dimerization is a consequence of activation of D2R. Both quinpirole (10 μM, 30 min), a specific D2R agonist, and dopamine (10 μM, 30 min) failed to up-regulate D2R dimerization (Figure 2-6 C-D); indicating that D2R activation alone is not sufficient to account for the amphetamine-induced up-regulation of D2R dimerization. More interestingly, amphetamine stimulation also failed to up-regulate D2R dimerization in HEK-293T cells transfected only with D2R, suggesting the involvement of additional proteins that exist in striatal neurons but not in transfected cells (Figure 2-7 A-B).

2.2.5 D2R-DAT protein-protein interaction is involved in amphetamine-induced D2R dimerization

Previous studies have shown that amphetamine increases dopamine concentration in the synaptic cleft by reversing DAT-mediated dopamine uptake. Based on the fact that both D2R activation alone and amphetamine stimulation in HEK-293T cells expressing D2R failed to up-regulate D2R dimerization, we speculate that DAT may be one of the additional proteins that
Figure 2-6. Amphetamine facilitates D2R dimerization in rat striatal neurons.

**A-B.** Western blot analysis of the expression levels of D2R dimers (A) and monomers (B) in primary cultures of rat striatal neurons treated with or without AMPH (30min, 10 μM).

***Significantly different from control group ($p<0.001$; n=5). Data were analyzed by $t$-test.

**C-D.** Western blot analysis of the expression levels of D2R dimers (C) and monomers (D) in primary cultures of rat striatal neurons treated with quinpirole (10 μM), dopamine (10 μM) or AMPH (10 μM). *, **Significantly different from control group ($p<0.05$, $p<0.01$; n=5). Data were analyzed by One-way ANOVA, followed by SNK post-hoc test.
Figure 2-7. DAT is involved in AMPH-induced D2R dimerization

A-B. Western blot analysis of the expression levels of D2R dimers (A) and monomers (B) in HEK-293T cells transfected with D2R in the presence or absence of AMPH (30min, 10 μM; n=3).

C-D. Western blot analysis of the expression levels of D2R dimers (C) and monomers (D) in HEK-293T cells co-expressing D2R/pcDNA3 or D2R/DAT in the presence or absence of AMPH (30min, 10 μM; n=3). *Significantly different from D2R/DAT control group (p<0.05; n=3). Data were analyzed by $t$-test.
play a role in this process. To explore whether the existence of DAT is necessary for D2R
dimerization, we examined the AMPH-induced D2R dimerization in HEK293T cells co-
expressing D2R and DAT, and in HEK293T cells co-expressing D2R and pcDNA3, a
mammalian expression vector in which DAT is subcloned. As shown in Figure 2-7 C-D, AMPH
failed to up-regulate D2R dimerization in the absence of DAT. We have previously reported that
the D2R forms a protein complex with DAT through direct protein-protein interactions [30].
Thus, if amphetamine induces up-regulation of D2R dimerization through a D2R-DAT
interaction, disruption of the D2R-DAT interaction with the interfering peptide TAT-DATNT1-1
encoding sequence of the interaction site of D2R-DAT within DAT [previously shown to disrupt
D2R-DAT coupling [30]] should block the amphetamine-induced up-regulation of D2R
dimerization. Consistent with our hypothesis, pre-treatment with TAT-DATNT1-1 peptide (30 min,
10 μM) blocked amphetamine-induced enhancement of D2R dimer formation (Figure 2-8 A-C),
while TAT-only and TAT-DATNT1-2 peptide (encodes scrambled sequence of TAT-DATNT1-1
peptide) showed no effect. The ability of TAT-DATNT1-1 peptide to disrupt D2R-DAT
interactions was confirmed in a parallel co-immunoprecipitation experiment (data not shown).
Taken together, these results suggest that D2R-DAT interaction may be necessary for the
amphetamine-induced up-regulation of D2R dimerization and that this interaction may also
contribute to the pathophysiology of schizophrenia.
Figure 2-8. D2R-DAT protein-protein interaction is involved in amphetamine-induced D2R dimerization.

**A.** Western blot analysis of the expression levels of D2R dimers and monomers in rat striatal slices treated with TAT-fused peptides (30min, 10μM) followed by amphetamine exposure (30min, 10μM).

**B-C.** Bar graphs summarizing the western blot data. *Significantly different from control group (p<0.05, n=3). Data were analyzed by One-way ANOVA, followed by SNK post hoc test.
2.3 Discussion

In summary, we have identified a potential role for D2R dimerization in the pathology of schizophrenia. This conclusion is based on our observations of enhanced expression of D2R dimers in postmortem human striatal sections of schizophrenia patients and in the striatum of an animal model of schizophrenia (AISS), as well as the acute amphetamine-induced up-regulation of D2R dimerization. Additionally, we provided evidence that the physical coupling between D2R and DAT may be necessary for this amphetamine-induced up-regulation of D2R dimerization.

It has been long recognized that D2R exists in both monomers and dimers in brain. However, the mechanisms regulating this process as well as the physiological/pathological roles of dimerization remain unknown. Our data provide the first direct evidence that both acute and chronic amphetamine treatments regulate D2R dimerization. Previous studies have shown that amphetamine enhances synaptic dopamine concentrations by the blockade and reversal of DAT-mediated dopamine uptake [31-33]. Thus, an obvious explanation may be that the enhanced synaptic dopamine level over-stimulates D2R and increases D2R dimerization. However, our results shown in Figure 2-6 C-D indicate that activation of D2R is not sufficient to induce D2R dimerization. We also observed that acute amphetamine treatment failed to induce D2R dimerization in HEK-293T cells expressing D2R only (Figure 2-7 A-B), suggesting the involvement of additional proteins/receptors in this process. Given that synaptic dopamine activates not only D2R but all dopamine receptor subtypes D1-D5, the observed amphetamine-induced up-regulation of D2R dimerization may also be involved in the activity of other dopamine receptors, such as D1R. Indeed, previous studies have shown a functional interaction between D1R and D2R, and the D1R and D2R may form a protein complex [34-36]. In addition
to D1R, recent studies have shown that D2R can also interact with other receptors or channels, such as D3R [37], Somatostatin sst5 receptor [38], Adenosine A2A receptor [39] and Kir3 K+ channel [40]. Several dopamine receptor interacting proteins (DRIPs) have also been identified to be directly and indirectly associated with D2R, including protein 4.1N/B/G [41], FilaminA [42], Spinophilin [43], GiPC [44], CAPS1 [45], ZIP [46], NCS-1 and GRK2 [47]. Future investigations may focus on studying the role of DRIPs as well as other D2R-interacting receptors in the AMPH-mediated D2R dimerization.

In many animal models of psychosis, the proportion of D2R in the high-affinity state is elevated 2- to 9-fold in the striatum of rats known to be supersensitive to dopamine [27, 48, 49]. Our current finding that amphetamine induces an increase in the proportion of D2^{High} receptors and an increase in the D2R dimers in rat tissue suggests that dimerization may be closely related to the dopamine-based hyperactivity elicited by amphetamine.

Previously, we reported that D2R directly interact with DAT, and the D2R-DAT protein complex formation enables D2R to up-regulate DAT-mediated dopamine uptake by recruiting DAT to the plasma membrane [30]. However, the role of D2R-DAT interaction in the regulation of D2R function remains unknown. In the current study, we provided evidence that disruption of the D2R-DAT interaction blocks amphetamine-induced up-regulation of D2R dimerization in transfected HEK293T cells, implicating the potential role of DAT in this process. However, it is worth noting that the AMPH–induced increase in D2R dimers seems to have bands that are more intense than the monomer bands. If the DAT is the only molecule required for this process, it may appear surprising that the DAT in such a small fraction of TH-positive neurons accounts for a large increase in DRD2 dimers in the cultured striatal neurons. Thus, there is a high probability that other additional molecules may be involved in D2R dimerization.
We have shown that D2R dimerization is enhanced in postmortem striatal sections from schizophrenia patients, and that amphetamine, a psychomotor stimulant that can induce psychosis, up-regulates D2R dimerization that in turn may be responsible for the noted dopamine hypersensitivity in schizophrenia. While the current antipsychotic treatments block dopamine D2 receptors, our data show that they do not alter the level of D2 dimers by themselves. Information from Stanley foundation indicated that 13 out of 15 patients in the schizophrenia group have received antipsychotic treatments and exhibited the increment of D2R dimerization. However, 12 out of 15 patients in the bipolar group have also received antipsychotic treatments but did not display an enhanced D2R dimerization. Therefore, a treatment focused at reversing the increased D2 dimerization my be closer to the pathophysiology of schizophrenia and may provide a novel therapeutic target for development of antipsychotics.

The advent of antipsychotic medications acting at D2R revolutionized the treatment of schizophrenia, primarily by alleviating positive symptoms. Based on the anti-dopaminergic properties of these drugs, one hypothesis proposed that the positive symptoms of schizophrenia are due to an excess of DA signaling in the striatal and/or mesolimbic areas of the brain through D2R. In contrast, negative symptoms are thought to be related to deficits in prefrontal cortical DA signaling, probably through D1R. In addition to schizophrenia, multiple sources of evidence have also supported a role for diminished dopaminergic neurotransmission in prefrontal cortex in major depression that may involve both D1R and D2R. We will continue to examine the role of dopamine receptors in the pathophysiology of depression in the next chapter.
2.4 Materials and Methods

2.4.1 Human postmortem brain tissue

Formalin-fixed paraffin-embedded human postmortem striatum sections (10μm-thick on glass slides) were donated by the Stanley Foundation Neuropathology Consortium [19]. Subjects were divided into four groups, including BD, MDD, SZ, and non-neurological/non-psychiatric controls (n = 15 per group). Subjects were matched for age, gender, postmortem interval (PMI), pH, and mRNA quality. Demographic and medical information such as drug abuse history and psychotropic treatments were provided by the Stanley Foundation Neuropathology Consortium. Diagnoses were retrospectively established by two senior psychiatrists using DSM-IV criteria. All experiments were performed blinded to the diagnosis of each subject.

2.4.2 Animals

Adult male Sprague–Dawley rats weighing 250–275 g were procured from Charles River Laboratories, Montreal, Canada. Animals were maintained on a 12-h light/dark cycle and housed two per cage with continuous access to food and water. The animals were allowed to acclimatize to the vivarium for a minimum of 5 days before being used for experimentation. All experimental protocols were approved by the CAMH animal care committee.

2.4.3 Chronic haloperidol treatment

Chronic haloperidol treatment was performed as previously described [20]. Briefly, groups of 12 rats each were randomly assigned to receive one of the following treatments: 0.25 mg/kg/day of haloperidol (McNeil Pharmaceuticals, Spring House, PA) or vehicle via Alzet osmotic mini-pumps (Alzet model 2ML4, Durect Corporation, Cupertino, CA) for a total of 2 weeks, to achieve continuous clinical occupancy.
2.4.4 Amphetamine-induced sensitized state

Amphetamine (Tocris Bioscience, Ellisville, MS) were given three times a week (Monday, Wednesday and Friday) for 5 weeks via intraperitoneal injection (IP). During week 1, amphetamine treated animals received a dose of 1 mg/kg (from salt); with the dose increasing by 1 mg/kg each week so that the dose in the final week was 5 mg/kg. Control animals received saline. All injections were administered in a 1 ml/kg volume.

2.4.5 Acute striatal slices

Acute striatal slices (350μm-thick) were prepared from Sprague-Dawley rats using a McIlwain tissue chopper (Mickle Laboratory Engineering, Gomshall, United Kingdom). Rat striata were dissected out and left for 5 min in ice-cold artificial cerebrospinal fluid (aCSF) containing 126mM NaCl, 2.5mM KCl, 1mM MgCl₂, 1mM CaCl₂, 1.25mM KH₂PO₄, 26mM NaHCO₃ and 20mM glucose, that was bubbled continuously with carbogen (95% O₂/5% CO₂) to adjust the pH to 7.4. Freshly cut slices were placed in an incubating chamber with carbogenated aCSF and recovered from stress at 37°C for 1 hour. Slices were then treated with 10 μM AMPH for 30 min. Slices were then harvested for Western blot analysis.

2.4.6 Primary cultured striatal neurons

Primary cultures from striatum were prepared from fetal Wistar rats (embryonic day 17-19) on culture dishes as previously described [21]. The cultures were used for experiments 12-15 days after plating.

2.4.7 HEK293T cell culture conditions and transfection

HEK293T cells were cultured in α-MEM (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Invitrogen) and maintained in incubators at 37°C, 5% CO₂. One day
before transfection, cells were split onto poly-D-lysine coated plates. For lipofectamine2000 transfections, DAT: D2R cDNA ratio of 1:5 to maximize coexpression. Cells were utilized 2 days post transfection.

2.4.8 Co-immunoprecipitation

Co-immunoprecipitation was performed as previously described [21, 22]. Briefly, solubilized rat striatal extracts (500~700 μg) were incubated in RIPA buffer with primary antibody anti-D2 (Millipore, Billerica, MA) or rabbit IgG (1~2 μg, Sigma-Aldrich, St. Louis, MO) for 4 h at 4°C, followed by the addition of 20 μl of protein A/G agarose (Santa Cruz, Santa Cruz, CA) for 12 h. Pellets were washed, boiled for 5 min in SDS sample buffer and subjected to SDS-PAGE. 20~50 μg of tissue extracted protein was used as a positive control in each experiment. Anti-DAT (Santa Cruz) was applied as the immunoblotting antibody in the following western blot analyses.

2.4.9 Western blot

Western blot analyses were performed as previously described [21, 22]. Briefly, formalin-fixed paraffin-embedded frozen human postmortem striatal sections from the Stanley Consortium (scraped off the glass slides), rat striatum, HEK293T cells and cultured rat striatal neurons (~2 x 10⁷) were homogenized in RIPA buffer (50mM Tris-Cl, pH 7.6, 150mM NaCl, 2mM EDTA, 1mM PMSF plus 1% Igepal CA-630, 0.5% sodium deoxycholate, 1% Triton X-100) with a protease inhibitor cocktail (Sigma-Aldrich), and centrifuged at 4 °C at 13,000 rpm for 10 min. Supernatant was extracted and protein concentrations were measured (Bio-Rad, Hercules, CA). Protein samples (50 μg) were boiled for 5 min in SDS sample buffer, and subjected to SDS-PAGE. Blots were blocked with 5% non-fat dried milk dissolved in TBST buffer (10 mM Tris, 150 mM NaCl, and 0.1% Tween 20) for 1 h at room temperature, washed three times with TBST
buffer, incubated with the appropriate primary antibody [anti-D2 (polyclonal) or anti-α-tubulin (monoclonal, Sigma-Aldrich) diluted in 5% milk in TBST] overnight at 4°C. The blots were washed again with TBST buffer three times and then incubated with horseradish peroxidase-conjugated secondary antibody (diluted in 5% milk in TBST; Sigma-Aldrich) for 1.5 h at room temperate. The proteins were visualized with enhanced chemiluminescence reagents (GE Healthcare, Piscataway, NJ).

2.4.10 Inhibition of [3H]-domperidone binding to D2R

The striatal tissue was homogenised (final concentration of 4 mg/ml) in a buffer containing 50 mM Tris-HCl (pH 7.4 at 20°C), 1 mM EDTA, 5 mM KCl, 1.5 mM CaCl₂, 4 mM MgCl₂ and 120 mM NaCl, using a Teflon-glass homogeniser with the piston rotating at 500 RPM and 10 up-and-down strokes of the glass container. Similar results were obtained with either washed or non-washed homogenates. Although it is known that approximately half the D2R can be lost upon washing the tissue [23], the homogenates were washed by centrifuging the homogenate at 10,000 x g for 10 min and discarding the supernatant; this procedure was repeated two more times. The final pellet was used.

The D2R in the striatal tissue were measured with [3H]-domperidone (2 nM final concentration; custom synthesized as [phenyl-3H(N)]-domperidone; 41.4 Ci/mmol; Moravek Radiochemicals Inc., Brea, CA) [24]. Each incubation tube (12 x 75 mm, glass) received, in the following order, 0.5 ml buffer, containing a range of dopamine concentrations, with or without a final concentration of 10 µM S-sulpiride (to define nonspecific D2R binding), 0.25 ml [3H]domperidone (generally 1.8 nM as the final concentration in the incubation tube), and 0.25 ml of tissue homogenate. Each concentration of dopamine was tested in duplicate. The tubes, containing a total volume of 1 ml, were incubated for 2 h at room temperature (20°C), after
which the incubates were filtered, using a 12-well cell harvester (Titertek, Skatron, Lier, Norway) and buffer-presoaked glass fiber filter mats (Whatman GF/C). After filtering the incubates, the filter mat was rinsed with buffer for 15 s (7.5 ml buffer), and the filters were processed as detailed above. The specific binding of $[^3$H]-domperidone was defined as total binding minus that in the presence of 10 µM S-sulpiride. Independent saturation of D2R, using a range of $[^3$H]-domperidone concentrations, revealed a $[^3$H]-domperidone dissociation constant (Kd) of $0.48 \pm 0.08$ nM (n = 6) for the rat homogenized striata.

2.4.11 TAT peptides pre-treatment

The acute rat striatal slices were pre-treated with TAT-fused peptides (30min, 10µM) before amphetamine treatment. TAT-fused peptides were synthesized by GeneScript. Peptides are rendered cell permeant by fusing to the cell membrane transduction domain of the human immunodeficiency virus type 1 TAT protein (YGRKKRRQRRR), as previously described [25].

2.4.12 2.5.12 Densitometry and statistical analysis

To quantify the bands obtained via Western blot analysis, we applied ImageJ software based analysis (http://rsb.info.nih.gov/ij/). The area under curve (AUC) of the specific signal was corrected for the AUC of the loading control (e.g. $\alpha$-tubulin). All values are provided as means ± SEM. For comparisons between two groups, t-test (two-tailed) was performed. For comparisons of more than two groups, one-way ANOVA followed by SNK post-hoc analysis was performed. Unless otherwise noted, significance level was set at 0.05.
References


Chapter 3

Uncoupling Dopamine D1-D2 Receptor Complex

Exerts Antidepressant-like Effects

Published previously as:


*These authors contribute equally to this work

Contributions of other authors:

I performed all experiments describe in the paper with equal contribution from Dr. Shupeng Li and Dr. Lin Pei

I performed the experiments in Figure 3-2 C-D, Figure 3-3 A and C, Figure 3-4, Figure 3-7 and Figure 3-8 B-E.
3.1 Introduction

Dopamine is a neurotransmitter that exerts complex actions and plays a crucial role in motor function, learning and memory, motivation and reward [1]. Disturbances of dopaminergic neurotransmission may lead to a variety of psychiatric diseases [1]. Successful clinical treatment of schizophrenia with D2R antagonists suggests a crucial role for dopamine in the pathophysiology of that disorder [2]. Furthermore, decreased dopaminergic signaling in depression has been suggested by observations of decreased levels of homovanillic acid, a major dopamine metabolite, in the cerebrospinal fluid (CSF) of depression patients [3] and by the successful use of the dopamine uptake inhibitor bupropion as an selective serotonin reuptake inhibitor (SSRI) augmentation strategy for treatment resistant patients [4].

The molecular actions of dopamine are mediated by five distinct receptor subtypes, some of which exist in different protein isoforms attributable to alternative RNA splicing. These receptors belong to the G-protein-coupled receptor (GPCR) superfamily and are divided into two major subgroups, D1-like and D2-like, on the basis of their structure, pharmacology, and transductional properties. Each type of these receptors displays its unique set of properties with respect to affinity for DA, potential for alternative splicing, and specificity of coupling to heterotrimeric GTP-binding G proteins. For instance, the D1-like subfamily is composed of the D1 and D5 subtypes, both of which transduce their signals by increasing intracellular cyclic AMP (cAMP) levels. The D2-like subfamily consists of the D2, D3, and D4 subtypes, all of which can diminish cAMP production and regulate calcium and potassium ion channels [1]. Previous animal studies showed that both D1R and D2R agonist mimic antidepressant effects while both D1R and D2R antagonists block the action of antidepressants [5-11]. However, the lack of clinical evidence for the effectiveness of either D1R or D2R agonists for the treatment of
depression has led to uncertainty concerning the role of D1R and D2R in the pathology of depression.

Recent findings revealed that D1R and D2R could form a receptor complex [12-14]. The D1-D2 receptor complex induces calcium release via a Gq-dependent pathway distinct from Gs/olf or Gi/o-dependent pathway activated by the D1R or D2R independently [13, 14]. The calcium mobilization activates CaMKII\(\alpha\) leading, in turn, to enhanced BDNF expression [15]. Although the pathophysiological role of the D1-D2 receptor complex remains elusive, results from work with other receptor complexes have shown that abnormal receptor-receptor coupling may play a critical role in human diseases [16, 17].

Thus, given that both D1R and D2R have been associated with mental illness and that calcium signaling plays a critical role in maintaining normal brain function, we hypothesized that D1-D2 receptor complex may play an important role in the pathophysiology of depression.
3.2 Results

3.2.1 D1-D2 receptor complex formation in postmortem striatal sections from depression patients

We initiated our investigation by carrying out co-immunoprecipitation experiments using a primary antibody against D2R on 30 post-mortem brain striatum samples from the Stanley Foundation. The material included 15 samples from each of two groups: control and severe depression. The two groups were matched by age, sex, race, postmortem interval, pH, side of brain, and mRNA quality control by the Stanley Foundation brain bank. The information regarding the antidepressant use for the patients suffering severe depression is listed in Table 1. Additional information regarding these samples is listed in Table 2. The same amount of protein from each sample was incubated with anti-D2R antibody and protein A/G agarose. The precipitated proteins were divided equally into two groups before being subjected to SDS-PAGE and immunoblotted with either D1R antibody or D2R antibody. Each Western blot included 3 samples from each group and the intensity of each protein band was quantified by densitometry (AIS software, Imaging Research Inc). Each sample is presented as the percentage of the mean of three control samples on the same blot. As shown in Figure 3-1 A, co-immunoprecipitation of D1R by the D2R antibody was significantly enhanced in the severe depression post-mortem brain samples compared to control brains (n=15, P< 0.05). The levels of directly immunoprecipitated D2R were not significantly different between control and severe depression groups (Figure 3-2 B). Furthermore, we measured the amount of D1R co-immunoprecipitated by anti-D2R primary antibody vs. the total D1R in an amount of striatal protein equal to that used in the co-immunoprecipitation experiment. As shown in Figure 3-1 C, the ratio of D1-D2 vs. D1R
Table 1: Information regarding antidepressant drug use for the major depression patient sample.

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>P-1</td>
<td>On amitriptyline. In past, nortriptyline.</td>
</tr>
<tr>
<td>P-2</td>
<td>On trazadone.</td>
</tr>
<tr>
<td>P-3</td>
<td>On fluoxetine.</td>
</tr>
<tr>
<td>P-4</td>
<td>Recent fluoxetine and trazadone.</td>
</tr>
<tr>
<td>P-5</td>
<td>Untreated for 6 years.</td>
</tr>
<tr>
<td>P-6</td>
<td>Past sertraline but not recent.</td>
</tr>
<tr>
<td>P-7</td>
<td>Recent fluoxetine.</td>
</tr>
<tr>
<td>P-8</td>
<td>On nefazadone.</td>
</tr>
<tr>
<td>P-9</td>
<td>Never treated.</td>
</tr>
<tr>
<td>P-10</td>
<td>Buspirone and imipramine.</td>
</tr>
<tr>
<td>P-11</td>
<td>Recent sertraline.</td>
</tr>
<tr>
<td>P-12</td>
<td>On buspirone and venlafaxine.</td>
</tr>
<tr>
<td>P-13</td>
<td>On nortriptyline and clomipramine.</td>
</tr>
<tr>
<td>P-14</td>
<td>Recent fluoxetine and amitriptyline.</td>
</tr>
<tr>
<td>P-15</td>
<td>On fluoxetine and nefazadone.</td>
</tr>
</tbody>
</table>
Table 2: General information on the 30 brain samples from the Stanley Foundation.

<table>
<thead>
<tr>
<th>Diagnosis (n=15 each)</th>
<th>Control</th>
<th>Depression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>48±11</td>
<td>47±9</td>
</tr>
<tr>
<td>Sex</td>
<td>6 female 9 male</td>
<td>6 female 9 male</td>
</tr>
<tr>
<td>Onset of disease (age)</td>
<td>N/A</td>
<td>34±13</td>
</tr>
<tr>
<td>Disease duration (yrs)</td>
<td>N/A</td>
<td>13±11</td>
</tr>
<tr>
<td>pH</td>
<td>6.3±0.2</td>
<td>6.2±0.2</td>
</tr>
<tr>
<td>PMI (h)</td>
<td>24±10</td>
<td>27±11</td>
</tr>
<tr>
<td>Psychosis present (n)</td>
<td>N/A</td>
<td>0</td>
</tr>
<tr>
<td>Suicide (n)</td>
<td>N/A</td>
<td>7</td>
</tr>
<tr>
<td>History of Substance Abuse (n)</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>Severity of Substance Abuse</td>
<td>2 lowest 1 low</td>
<td>1 lowest 1 lower 1 higher 2 highest</td>
</tr>
<tr>
<td>History of Alcohol Abuse (n)</td>
<td>15</td>
<td>14</td>
</tr>
<tr>
<td>Severity of Alcohol Abuse</td>
<td>5 lowest 6 lower 2 low 2 high</td>
<td>4 lowest 5 lower 1 high 1 higher 3 highest</td>
</tr>
<tr>
<td>Side (Striatum)</td>
<td>8 left 7 right</td>
<td>9 left 6 right</td>
</tr>
</tbody>
</table>
Figure 3-1. D1-D2 receptor interaction is enhanced in postmortem striatal tissue of depression patients.

Striatal post-mortem brain samples (control and major depression; 15 samples in each group), obtained from the Stanley Foundation, were incubated with D2R antibodies for coimmunoprecipitation experiments. Precipitated proteins were subject to SDS-PAGE; immunoblotted with either D1R antibody or D2R antibody.

A. Co-immunoprecipitation of D1R by the D2R antibody is significantly increased in major depression brains compared to controls (* P< 0.05, n=15, t-test).

B. The level of immunoprecipitated D2R is not significantly altered in major depression brain samples.

C. The ratio of the D1R co-immunoprecipitated by D2R antibody vs. the total D1R in an equal amount of striatal protein used in the co-immunoprecipitation experiment was significantly increased in major depression group compared to control group. (* P< 0.05, n=15, t-test).
was significantly enhanced in the severe depression group compared to the control group (n=15, p< 0.05). These data suggest enhanced D1-D2 complex formation in the post-mortem brain samples of patients suffering from severe depression.

3.2.2 D1 and D2 receptors form a direct protein-protein interaction via D1CT and D2IL3

We then reasoned that if enhanced D1-D2 coupling plays a role in the pathophysiology of depression, disruption of D1-D2 coupling might exert antidepressant effects. In order to develop a protein peptide able to disrupt D1-D2 coupling, we performed a series of biochemical analyses to identify regions of the D1R and D2R that are important for D1-D2 complex formation.

Various glutathione-S-transferase (GST) fusion proteins encoding the third intracellular loop (IL3) and/or CT of D1R (GST-D1IL3 [R219-K272], GST-D1CT [A332-T446], D5 receptor (GST-D5CT [A360-H477]) and two molecular isoforms of D2R termed D2Long (D2L) and D2Short (D2S), arising from alternative splicing (GST-D2LIL3 [K211-Q373], GST-D2SIL3 [K211-Q344], GST-D2CT [T399-C414]), were prepared and utilized in affinity purification assays. As shown in Figure 3-2 A, GST-D2LIL3, but not GST-D2SIL3, GST-D2CT or GST alone, precipitated D1R from solubilized rat striatum indicating that D1R can interact with D2R via the third intracellular loop of D2L.

The fact that D2LIL3 differs from D2SIL3 by an additional 29 amino-acids in the third intracellular loop (D2IL3-29 [N243-E271]) [4], and the fact that GST-D2LIL3, but not GST-D2SIL3, was able to affinity “pull down” D1R suggested that D1R might interact with D2R via the 29 amino-acid segment. Consistent with our hypothesis, GST-D2IL3-29 was able to “pull down” D1R (Figure 3-2 B) and further experiments indicated that the region of M257-E271 (D2IL3-29-2), but not N243-I256 (D2IL3-29-1) of the D2IL3-29 can successfully pull-down D1R from solubilized rat striatum (Figure 3-2 B). Using the same strategy, we concluded that D1R interacted with D2R through the D1CT, since GST-D1CT, but not GST-D1IL3 or GST-D5CT was able to precipitate D2R (Figure 3-2 C).
Figure 3-2. Biochemical association of D1-D2 receptor complex.

A-B. D1R was specifically pulled down from solubilized rat striatal extracts by GSTD2L_{IL3} (A) and GST-D2_{IL3-29}, GST-D2_{IL3-29-2} (B).

C. D2R was affinity purified from solubilized rat striatum with GST-D1_{CT}.

D. In vitro binding assay depicting the [35S]-D1CT binding with GST-D2_{IL3-29} and GST-D2_{IL3-29-2}. 
Although these results demonstrate the presence of the D1-D2 complex in rat striatal tissue, they did not clarify whether the D1-D2 complex is formed through a direct interaction between D1R and D2R or mediated by an indirect interaction involving accessory binding proteins. In vitro binding assays demonstrated that [\(^{35}\text{S}\)]-D1CT hybridized with GST-D2L-IL3-29-2, but not with GST-D2L-IL3-29-1, suggesting the possibility of a direct D1-D2 interaction via the D2L-IL3-29-2 region (Figure 3-2 D). To further confirm the important role of the D2L-IL3-29-2 sequences in enabling the D1-D2 interaction, we sought to perturb the D1-D2 interaction by introducing an interfering protein peptide encoding D2L-IL3-29-2. D2L-IL3-29-2 and D2L-IL3-29-1 peptides were rendered cell-permeant by fusing each to the cell-membrane transduction domain of the human immunodeficiency virus-type 1 (HIV-1) TAT protein (Tyr-Gly-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Arg) [18]. As shown in the co-immunoprecipitation experiment in Figure 3-3 A-B, using the protein extracted from rat striatum, both the ability of the D1R primary antibody to co-immunoprecipitate D2R and the ability of the D2R primary antibody to co-immunoprecipitate D1R are significantly decreased by co-incubation with the purified TAT-D2L-IL3-29-2 peptide, but not with the TAT-D2L-IL3-29-1 peptide or TAT alone. We also confirmed the result in rat prefrontal cortex (PFC) tissue since both brain regions have been implicated in the pathophysiology of depression (Figure 3-3 C-D) [19]. Furthermore, we also examined whether disruption of D1-D2 complex formation will block functional interaction between D1R and D2R. As shown in Figure 3-4 A, pre-incubating HEK-293T cells expressing D1R and D2R with TAT-D2L-IL3-29-2 (10 \(\mu\text{M}, 30\text{ min}\)), but not TAT alone, blocked the co-activation of D1R- and D2R-induced calcium release. Taken together, these data support the existence of a direct protein-protein interaction occurring between D1R and D2R and confirm the role of M\(_{257}\)-E\(_{271}\) region of D2R in maintaining the D1-D2 direct protein-protein interaction.
Figure 3-3. Essential role of the D2L_{IL3-29-2} in enabling the D1-D2 interaction.

*A-B.* The ability of D1R antibody to co-immunoprecipitate D2R (A) and D2R antibody to co-immunoprecipitate D1R (B) from rat striatum (STR) was significantly inhibited in the presence of TAT-D2L_{IL3-29-2}.

*C-D.* The ability of D1R antibody to co-immunoprecipitate D2R (C) and D2R antibody to co-immunoprecipitate D1R (D) from rat prefrontal cortex (PFC) was significantly inhibited in the presence of TAT-D2L_{IL3-29-2}.
Figure 3-4: Disruption of D1-D2 coupling blocked the co-activation of D1R/D2R induced calcium release.

A. In HEK-293T cells expressing D1R and D2R, pre-incubating the cells with TATD2IL3-29-2 (10 μM, 30 min), but not TAT alone, significantly inhibited the calcium response co-activated by 10 μM (20 min) each of SKF 81297 (specific D1R agonist) and quinpirole (Quin, specific D2R agonist). **Significantly different from the control group (n=5, P<0.01). #Significantly different from the SKF + Quin group (n=5, P<0.05). SKF: SKF81297; Quin: Quinpirole. Data were analyzed by one-way ANOVA.
3.2.3 Disruption of D1-D2 interaction exerts anti-depressant-like effects

Having shown that D1-D2 interaction is up-regulated in postmortem brain tissue of depressed individuals, and having identified an interfering peptide that can disrupt the D1-D2 interaction, we then investigated whether disrupting the D1-D2 interaction through infusions of the TAT-D2L-IL3-29-2 peptide into the PFC bilaterally has antidepressant-like effects in a forced swim test (FST). As shown in Figure 3-5 A, TAT-D2L-IL3-29-2 peptide, but not TAT-D2L-IL3-29-1 peptide, caused a significant reduction of immobility (p<0.01, n=9), an effect comparable to that induced by the antidepressant imipramine. To exclude the possibility that TAT-D2L-IL3-29-2 PFC infusions may induce nonspecific motor changes in the FST, we measured TAT-D2L-IL3-29-2 effects on locomotor activity. As shown in Figure 3-5 B, rats injected with TAT-D2L-IL3-29-2 displayed similar level of ambulatory behavior compared to rats injected with TAT-D2L-IL3-29-1 or saline, suggesting some specificity of the antidepressant-like effects of TAT-D2L-IL3-29-2.

Furthermore, infusion of TAT-D2L-IL3-29-2 peptide into the hippocampus or nucleus accumbens did not exert antidepressant effect Figure 3-6 A or nonspecific motor effect Figure 3-6 B. To confirm that the observed antidepressant-like effects resulted from disruption of the D1-D2 interaction, we tested the D1-D2 association in the brain of rats exposed to FST. As shown in Figure 3-7 A, the co-immunoprecipitation of D1R by the D2R antibody was significantly decreased in the FST rats injected with TAT-D2L-IL3-29-2 compared to FST rats injected with TAT-D2L-IL3-29-1 (n=6, P<0.01). The levels of directly immunoprecipitated D2R were not significantly different between these groups (Figure 3-7 B).

Because the FST is an acute test of antidepressant activity, we also tested the potential antidepressant effects of TAT-D2L-IL3-29-2 peptide in the 5-day learned helplessness (LH) paradigm [20]. We initiated our investigation by examining whether the D1-D2 association was
**Figure 3-5.** TAT-D2 IL3-29-2 treatment in PFC exerts antidepressant-like effects in FST.

**A.** Rats receiving bilateral PFC TAT-D2 IL3-29-2 infusions showed significantly reduced immobility and increased swimming time. **Significantly different from the TAT-D2 IL3-29-1 group (P<0.01, n=9). #Significantly different from the saline group (P<0.05, n=9).** Data were analyzed by one-way ANOVA followed by post-hoc LSD test.

**B.** Levels of ambulatory activity in rats infused with TAT-D2IL3-29-1 or TAT-D2IL3-29-2 in the PFC or injected with imipramine (IP).
Figure 3-6. Effects of TAT-D2IL3-29-1 treatment in other brain regions in FST.

A. Rats receiving TAT-D2IL3-29-2 infusion in the hippocampus or nucleus accumbens (N.Acc) showed no changes in immobility and swimming time compared to rats that received TAT or TAT-D2IL3-29-1 infusions in PFC.

B. Levels of ambulatory behavior in rats infused with TAT, TAT-D2IL3-29-1 or TATD2IL3-29-2 in the PFC, hippocampus and N.Acc. # $P<0.05$, ** $P<0.01$, $n=7-9$. Data were analyzed by one-way ANOVA followed by post-hoc SNK tests.
**Figure 3-7. The D1-D2 association in the brain of rats exposed to FST.**

**A.** Co-immunoprecipitation of D1R by the D2R antibody was significantly decreased in PFC of FST rats injected with TAT-D2IL3-29-2 compared to FST rats injected with TAT-D2IL3-29-1. **Significantly different from the TAT-D2IL3-29-1 group, \( P<0.01, n=6 \). **

\( \#\# P<0.01; \### P<0.001, \) significantly different from the saline group, \( n=6 \). Data were analyzed by one-way ANOVA followed by post-hoc SNK tests.

**B.** Level of D2R immunoprecipitated by the D2R antibody in PFC of FST rats injected with TAT-D2IL3-29-1, TAT-D2IL3-29-2 or imipramine.
altered in the brain tissue of rats that had developed learned helplessness. As shown in Figure 3-8 A, rats that received inescapable foot shock showed a significantly elevated number of escape failures, which could be blocked by imipramine treatment. Consistent with our hypothesis, an enhanced D1-D2 association was observed in both PFC and striatum of rats that displayed enhanced escape failure (Figure 3-8 B-C). The levels of directly immunoprecipitated D2R were not significantly different among these groups (Figure 3-8 D-E). Furthermore, as shown in Figure 3-8 F-G, the ratio of D1-D2 vs. D1R was also significantly enhanced in the stress group compared to the control group. We then tested whether disruption of D1-D2 association would block the observed enhancement of escape failures by intracerebroventricular (ICV) injection of TAT-D2L-IL3-29-2 peptide. We chose to use the ICV route instead of PFC injection due to the requirement of repeated injections in the LH model. As shown in Figure 3-9 A, TAT-D2L-IL3-29-2 peptide, but not TAT-D2L-IL3-29-1 peptide, significantly reduced escape failures in rats subjected to inescapable foot shock, just as an antidepressant, such as imipramine, had such an effect. Taken together, these data strongly suggest that disruption of D1-D2 association by the TAT-D2L-IL3-29-2 peptide may exert antidepressant effects.
Figure 3-8. TAT-D2IL3-29-2 treatment exerts antidepressant-like effects in LH model.

**A.** Rats receiving inescapable foot shock (IES) displayed significantly elevated escape failures compared to cage control rats, which was blocked by imipramine. * $P<0.05$; ** $P<0.01$; significantly different from the cage control group, $n=14$, two-way repeated ANOVA followed by Holm-Sidak test;

**B-C.** Co-immunoprecipitation of D1R by the D2R antibody was significantly increased in rats receiving IES compared to the cage control group in rat PFC (B) and Striatum (STR) (C). This stress-mediated effect was blocked by imipramine. **, $P<0.01$; *** $P<0.001$; Significantly different from the control group, $n=4$. ## $P<0.01$; # $P<0.05$; Significantly different from the stress group, $n=4$. Data were analyzed by one-way ANOVA followed by post-hoc SNK tests.

**D-E.** Level of D2R immunoprecipitated by the D2R antibody in PFC (D) and Striatum (STR) (E) of LH rats (cage controls, stress and imipramine groups).

**F-G.** The ratio of D1R co-immunoprecipitated by the D2R antibody vs. total D1R in an equal amount of protein as that used in the co-immunoprecipitation was significantly increased in rats receiving IES compared to cage controls in rat PFC (f) and STR (g). This stress-mediated effect was blocked by imipramine. * $P<0.05$; *** $P<0.001$; Significantly different from the control group, $n=4$. # $P<0.05$, ### $P<0.001$; Significantly different from the stress group, $n=4$. Data were analyzed by one-way ANOVA followed by post-hoc SNK tests.
Figure 3-9. TAT-D2IL3-29-2 significantly reduced escape failures in rats subjected to inescapable foot shock.

A. Administration of TAT-D2IL3-29-2 (ICV) and intraperitoneal (IP) imipramine in rats receiving IES resulted in a significantly decreased number of escape failures compared to rats administrated ICV TAT-D2IL3-29-1. Rats receiving IP imipramine also received ICV saline. ** $P<0.01$; significantly different from the TAT-D2IL3-29-1 group, $n=19$. Two-way repeated ANOVA followed by Holm-Sidak test.
3.3 Discussion

In summary, our study provides the first direct evidence implicating the D1-D2 protein complex in the pathology of depression, and also identifies an interfering protein peptide that can disrupt the D1-D2 interaction and exert antidepressant-like effects.

Major depressive disorder is an illness associated with significant morbidity that may lead to substantial impairment in functioning. With current clinical antidepressant treatments, only 1/3 of patients achieve full remission of their symptoms after a single trial of antidepressant medications. Even with multiple antidepressant trials, 10-15% of patients continue to experience persistent depressive symptoms and few alternatives have been available for the treatment of resistant symptoms [21]. Thus, alternative novel antidepressant treatments are needed. The identification of D1-D2 interaction interfering peptide with antidepressant activity may provide a new therapeutic strategy for the treatment of major depression disorder.

3.4 Materials and Methods
3.4.1 GST Fusion Proteins and Mini-genes

To construct GST-fusion proteins expressing truncated D1CT, D1IL3, D2CT, D2LIL3, D2SIL3, D2IL3-29, D2IL3-29-1, D2IL3-29-2 and D5CT, cDNA fragments were amplified by using PCR with specific primers. Except where specified, all 5’ and 3’ oligonucleotides incorporated BamH1 site (GGATCC) and Xho1 sites (CTCGAG), respectively, to facilitate subcloning into vector pGEX-4T3 (for GST-fusion protein construction) or PCDNA3 (for transfection). GST-fusion proteins were prepared from bacterial lysates as described by the manufacturer (Amersham). To confirm appropriate splice fusion and the absence of spurious PCR generated nucleotide errors, all constructs were resequenced.
3.4.2 GST fusion Protein affinity purification, co-immunoprecipitation and Western blotting

Co-immunoprecipitation, GST affinity pull-down and Western blot analyses were performed as previously described [22, 23]. For co-immunoprecipitation experiments, solubilized striatal/cell extracts (500-800 \( \mu \text{g} \)) protein were incubated in the presence of primary antibody anti-D2 (Chemicon), anti-D1 (Sigma-Aldrich) or rabbit IgG (Sigma-Aldrich) (1~2 \( \mu \text{g} \)) for 4 h at 4\(^{\circ}\)C, followed by the addition of 20 \( \mu \text{l} \) of protein A/G agarose (Santa Cruz) for 12 h. Pellets were washed, boiled for 5 min in SDS sample buffer and subjected to 10\% SDS-PAGE. For Western blot analysis, 50 \( \mu \text{g} \) of tissue-extracted protein was used as a control in each experiment. For affinity purification experiments, solubilized tissue extracts (800 \( \mu \text{g} \) protein) were incubated with glutathione-sepharose beads (GE Healthcare) bound to the indicated GST-fusion proteins (50\( \mu \text{g} \)) at room temperature for 1 hour. Beads were washed, boiled for 5 min in SDS sample buffer and subjected to SDS-PAGE. After transfer of proteins onto nitrocellulose, membranes were Western blotted with either the monoclonal D1 antibody (Sigma-Aldrich) or polyclonal D2 antibody (Santa Cruz Biotechnology), or polyclonal D2 antibody (Chemicon). For competitive co-immunoprecipitation, briefly, solubilized tissue extracts (800 \( \mu \text{g} \) of protein) were incubated with purified protein peptides TAT, TAT-D2\(_{\text{IL3-29-1}}\) or TAT-D2\(_{\text{IL3-29-2}}\) in the presence of primary antibody D1R (D-187; Sigma-Aldrich) for 4 hours at 4\(^{\circ}\), followed by the addition of 20 \( \mu \text{l} \) of protein A/G agarose (Santa Cruz Biotechnology) for 12 hours. Pellets were washed, boiled for 5 min in SDS sample buffer and subjected to 10\% SDS-PAGE. 50 \( \mu \text{g} \) of striatal tissue extracted protein was used as control in each experiment.
3.4.3 In vitro binding assays

Glutathione beads carrying GST fusion proteins (D2\textsubscript{IL3-29}, D2\textsubscript{IL3-29-1} and D2\textsubscript{IL3-29-2}) or GST (10 to \(\sim\)20 \(\mu\)g each) alone were incubated with \(^{[35}\text{S}]-\text{methionine-labeled D1-CT}\) respectively. Then the beads were washed six times with PBS containing 0.5% Triton X-100 and eluted with 10 mM glutathione elution buffer. Eluates were separated by SDS-PAGE and visualized by autoradiography.

3.4.4 Animals

Adult Male Sprague Dawley rats (Charles River Laboratories, Wilmington, MA) were used in experiments. Rats were housed at a constant temperature (20–23 °C), illumination (12-h light/12-h dark cycle, light on at 08:00 h) with food and water \textit{ad libitum}. All experimental procedures were approved by the Animal Committee of the Centre for Addiction and Mental Health.

3.4.5 Surgical procedure and drug/peptide treatment in FST

Surgery took place 1 week after arrival of the animals in the laboratory. Bilateral prefrontal cortical cannulation was performed under ketamine (50 mg/kg/IP) anesthesia. The top of the skull was shaved and swabbed with an antiseptic, after which a midline frontal incision was made in the scalp and the skin was retracted bilaterally. Burr holes (2 mm) were drilled into the skull and cannulae were implanted at the following coordinate: for prefrontal cortex: AP 2.2 mm, LM 0.8 mm, DV 4.0 mm to bregma; for dorsal hippocampus: AP \(-3.5\) mm, LM 2.4 mm, DV 3.0 to bregma; for nucleus accumbens at a 12° angle: AP 1.8 mm, LM 2.6 mm, DV 7.4 from dura to bregma. The rats were given 1 week to recover from the surgical procedure and were handled daily throughout the recovery period since the degree of irritability depends on the degree of
handling following surgery. Saline, peptide or imipramine treatment began one week after surgery and was given three times with the FST test: immediately after the 15 min training, 5 hr after the 15 min training and 24 hr after the training (1 hr before the FST test). Rats received 0.5 μl infusions of either TAT-D2IL3-29-1, TAT-D2IL3-29-2, saline or TAT peptide at a rate of 0.35 μl/min into PFC, hippocampus or NAC. The injection cannula was left in place for 1 min following the infusion and rats were returned to their home cages prior to commencing the test. 24 h after last treatment the animals were sacrificed by decapitation under isoflurane anesthetic and their brains were rapidly removed. Half of each brain was frozen on dry ice, and stored frozen (−40 °C) until assayed; the other half was stored in 4% formaldehyde for at least three days, after which it was transferred to a 10% sucrose solution for an additional day. Frozen sections of 30 μm were collected on glass slides coated with glycerine and stained with cresyl violet to verify the injection sites.

### 3.4.6 Forced swim test (FST)

This study was carried out in rats according to the methods first described by Porsolt and colleagues (1978) [24] and extensively used since then to detect antidepressant activity of various forms of interventions [25-28]. Briefly, rats were placed in Plexiglas cylinders (height: 40 cm, diameter: 18 cm) filled to a height of 25 cm with water maintained at 23–25°C. After 15 min in the water, they were removed, hand-dried with a towel and allowed 15 min in a heated container before being returned to their home cages. They were placed again in the cylinders 24 h later and the following behaviors were scored as the predominant one in 5-sec bins throughout a 5–min test: immobility, swimming, diving or climbing [25]. A rat was judged to be immobile when it remained floating passively in the water.
3.4.7 Open field test

All rats were subjected to an open field test 1hr after TAT, TAT-D2$_{IL3-29-1}$ or TAT-D2$_{IL3-29-2}$ administration. Locomotor activity assessment was carried out in 40 x 40 x 35 cm Plexiglas activity monitor chambers (MED Associates, St. Albans, VT.), which detect and record horizontal locomotor activity via infrared beam breaks. Locomotor activity was measured during a 30 minutes period.

3.4.8 Learned helplessness

We followed with minor modifications the basic 5-day protocol used by Soubrié and colleagues because it has been shown to be sensitive to a number of antidepressant interventions [20, 29, 30]. Briefly, on day 1 rats were placed in sound-attenuated operant boxes (Med Associates, St Albans, VT), where they received 0.8 mA inescapable shocks. Rats received approximately 100 randomly spaced shocks with durations between 10 and 20 seconds each (mean = 15 seconds), for a fixed total shock exposure of 25 min. Control rats remained in their home cages. Six hours after the inescapable shock trials rats were injected ICV with 20 mM of the target peptide (TAT-D2$_{IL3-29-1}$ or TAT-D2$_{IL3-29-2}$, total volume 4µL at a rate of 0.35µL/min). On day 2, TAT-D2$_{IL3-29-1}$ and TAT-D2$_{IL3-29-2}$ rats were injected twice with 10 mM of the appropriate peptide, 6 hr apart. Rats in the Imipramine group shown in Figure 2D received two intraperitoneal (IP) injections of 15 mg/kg at those times. Rats in the Imipramine group shown in Figure 2H were injected ICV with saline in addition to the two IP injections of Imipramine. On day 3 avoidance training began. Shuttle boxes (Med Associates) placed in sound- and light-attenuating chambers were divided into two compartments of equal size separated by a translucent partition with a single opening. A total of 30 trials were given each day, each trial consisting of 5 sec of the conditioned stimulus (CS, a continuous 90 dB tone), followed by 5 sec
of the CS+ the unconditioned stimulus (US, 0.8 mA foot shock). If the rat moved through the partition within the first 5 sec, it received no shock and the trial was recorded as a successful avoidance. Otherwise the trial would be recorded as a failure to avoid. If no crossing occurred the trial automatically ended after 5 sec of shock. One hour before the avoidance session, and again 6 hr thereafter, rats received ICV injections of the appropriate peptide or IP injections of imipramine. The procedure, including the injection regimen, was repeated on days 4 and 5.

3.4.9 Statistical analysis

Statistical analysis of the data was accomplished by means of the SPSS® statistical software package (SPSS). For the FST test, one-way analysis of variance (ANOVA) followed by Fischer’s protected least significant difference post hoc test. For learned helpless test, escape failures were examined by two-way repeated measures ANOVA with treatment as a between group factor and test day as a within group factor, followed by Holm-Sidak post hoc tests. For experiments with two groups, Student t-test was used. The level of statistical significance was set at p < 0.05. All results are expressed as mean ± SEM.

3.4.10 Measurement of Calcium Signal

Calcium mobilization assays were performed as previous described [4]. Briefly, the calcium signal was detected by the multi-well plated fluoro-meter (PerkinElmer). Transfected HEK293T cells co-expressing D1R and D2R pretreated with/without TAT or TAT-D2IL3-29-2 (10 μM, 30 min) were seeded in black 96-well plates and grown for 48 h after transfection. The cells were then loaded with 2 μM Fluo-4AM indicator dye (Molecular Probes, Inc., Eugene, OR) in growth medium supplemented with 20 mM HEPES and 2.5 mM probenecid for 1 h and subsequently washed twice with Hanks’ balanced salt solution without sodium bicarbonate and phenol red (Invitrogen). Baseline fluorescence values were measured for 15 s, and changes in
fluorescence corresponding to alterations in intracellular calcium levels upon the addition of agonists or antagonists thereafter were also recorded since 30 s. Fluorescence values were collected at 3-s intervals for 150 s.
References


Chapter 4

Essential Role of the GAPDH-GluR2 Interaction in Mediating Ischemic Neuronal Damage

Contributions of other authors:

I performed all experiments, unless specified, with equal contribution from Dr. Shupeng Li:

• Dr. Shupeng Li built the global ischemia model and performed experiments in focal ischemia model (Figure 4-18).
• Dr. Sheng Chen conducted the confocal experiment (Figure 4-10).
• Dr. Shengwei Zou performed GST-pull down experiment (Figure 4-1 A and B)
• Dr. Dongxu Zhai performed experiments in focal ischemia model with Dr. Shupeng Li.
• Dr. Lin Pei helped prepare the acute rat brain slices.
4.1 Introduction

Glutamate is the principal excitatory neurotransmitter in the brain and is involved in numerous physiological functions including neuronal circuit development, learning and memory [1]. It is also implicated in neuropathological disorders such as stroke and epilepsy and is also associated with neurotoxicity [2]. Glutamate activates two major subfamilies of ligand-gated ion channels: NMDAR and AMPAR [3]. AMPAR mediates fast synaptic transmission at excitatory synapses, while NMDAR is critical in producing a number of different forms of synaptic plasticity [1].

AMPA receptors were initially cloned as a kainite-gated ion channel by using the *Xenopus* oocyte expression system [4]. The first AMPA receptor subunit to be cloned is GluR1. Three other subunits of AMPA receptors are subsequently identified by PCR screenings based on sequence homologies. These subunits are later named as GluR1 to GluR4, or alternatively GluR A to GluR D [5, 6]. Each subunit has the same topology, consisting of three transmembrane (TM) domains and one membrane reentrant loop. The amino-terminus (NT) is the major extracellular domain and the carboxyl-terminus (CT) is the major intracellular domain [3]. The extracellular domain can be divided into three regions: (1) the N-terminal domain (NTD ~400 amino acids) (2) the S1 domain (~150 amino acids) immediately following the NTD and (3) the S2 domain (~150 amino acids) which is composed of the extracellular loop region between the 3\textsuperscript{rd} and 4\textsuperscript{th} TM domains. S1 and S2 domains have similar sequence homology with bacterial peri-plasmic amino acid-binding proteins. They are both necessary and sufficient for glutamate and other ligand binding [7]. The amino acid sequences of these domains are highly conserved among the four subunits, consistent with their importance in the core function of AMPA receptors. The NTD sequence is less conserved among different subunits, and its
function is still unclear, although it may be involved in the multi-merization of receptor subunits [8-10]. The CT of the AMPA receptor is the major intracellular domain and is important for receptor regulation. It is found in short or long forms, in terms of the length and homology. The long CT group contains GluR1, GluR2-long form (a splice variant of GluR2) [11], and GluR4. The short group contains GluR2, GluR3, and GluR4c (a splice variant of GluR4) [12, 13]. These two different groups of CT are important for AMPA receptor regulation.

In neurons, AMPA receptors form hetero-oligomers consisting of various combinations of GluR1 to GluR4 subunits. Mature receptors are found as tetramers [14]. Cloning of AMPA receptor subunits has allowed for their functional characterization. GluR1/3/4 homomers show inwardly rectifying current-voltage dynamics, while GluR2 homomers show a linear current-voltage relationship [6, 15]. GluR1/3/4 homomers are permeable to both Na\(^+\) and Ca\(^{2+}\) ions. Conversely, GluR2 homomers are uniquely impermeable to Ca\(^{2+}\) ions while conducting Na\(^+\) ions. This ion selectivity of GluR2 is dominant over other subunits in heteromeric AMPA receptors. The majority of AMPA receptors \textit{in vivo} contain the GluR2 subunit [6, 15]. While GluR4 subunits are expressed mainly in the early postnatal period [16]. These oligomeric combinations are assembled in the endoplasmic reticulum (ER) through mechanisms that are not well understood but seem to depend on interactions between the NTD of the subunits [8, 9]. GluR1/2 hetero-oligomers exit the ER rapidly, and traffic to the Golgi apparatus where they become fully glycosylated [17]. In contrast, GluR2/3 hetero-oligomers take much longer to exit. A fraction of the GluR2 subunits seems to reside stably within the ER as an intracellular and immature pool. This GluR2 pool seems to be actively retained within the ER and is dependent on the presence of a charged arginine residue (R607) at the channel pore region of the GluR2 subunit. This arginine residue is acquired through the RNA editing of the original sequence coding for glutamine [18]. GluR1/3/4 mRNAs are not edited at this position, and therefore, they
are not restrained within the ER. The retention protein that prevents immature GluR2 from exiting the ER is unknown, however, a fraction of AMPA receptors associates with ER chaperones Immunoglobulin Binding Protein (BiP) and calnexin [18], and GluR2 co-localizes extensively with BiP in the ER [17]. Therefore, it is possible that these chaperones or other proteins residing at the ER are related to the retention mechanisms.

The accumulation of glutamate, which occurs immediately after ischemia, results in excessive stimulation of glutamate receptors and leads to neurotoxicity [19, 20]. NMDAR-mediated neurotoxicity is dependent upon extracellular Ca\(^{2+}\) and is likely mediated by Ca\(^{2+}\) influx directly through receptor-gated ion channels [19, 20]. AMPAR is also tightly associated with a selective pattern of neuronal loss in certain brain areas following both global and focal ischemia [21-33]. Similar to what is reported for NMDARs, excitotoxicity mediated by AMPARs lacking the GluR2 subunit is thought to depend on ion influx (Ca\(^{2+}\), Zn\(^{2+}\)) through AMPAR channels following agonist stimulation [32-34]. However, as most native AMPARs in the hippocampus contain the GluR2 subunit and therefore are likely impermeable to Ca\(^{2+}\) [16, 17, 35-37], it is still unclear how the activation of GluR2 AMPAR leads to neuronal cell death.

Protein-protein interactions with AMPAR affect its function. The best characterized AMPAR interacting proteins interact with the intracellular carboxyl terminus of AMPAR. These proteins include GRIP (glutamate receptor-interacting protein), ABP (AMPA-binding protein), SAP97 (synapse-associated protein-97), PICK1 (protein interacting with C kinase-1), stargazin, NSF and AP2 (adaptor protein-2) [38-45]. These proteins regulate AMPAR function in a variety of ways, including altered AMPAR subcellular localization, clustering and/or trafficking. In addition, recent work has demonstrated that NARP (neuronal activity-regulated pentraxin) and
N-cadherin interact with the amino terminus (NT) of AMPAR subunits and play an important role in AMPAR clustering [46] as well as dendritic spine formation [47].

Given the fact that both GAPDH and AMPAR are involved in the cell death process, we hypothesized that GAPDH-GluR2 complex may play an important role in the AMPAR-mediated excitotoxicity.
4.2 Results

4.2.1 GluR2 subunit directly interacts with GAPDH via its N-terminal domain

To identify potential proteins that may interact with the NT of AMPAR subunits, we used GST-fusion proteins GST-GluR1<sub>NT</sub> (A<sub>19</sub>-E<sub>538</sub>) and GST-GluR2<sub>NT</sub> (V<sub>22</sub>-E<sub>545</sub>) to affinity purify “pull-down” proteins from solubilized rat hippocampal tissues along with GST alone as a control. The precipitated proteins were then identified by Coomassie brilliant blue staining following SDS-PAGE. A prominent protein band of ~37 kDa was specifically precipitated by GST-GluR2<sub>NT</sub>, but not by either GST alone or GST-GluR1<sub>NT</sub> (Figure 4-1A). Mass spectrometry analysis (LC-MS/MS, Protana [now Transition Therapeutics]) of this protein band identified three fragments that were homologous to and covered 17% of the sequences within rat GAPDH (VIISAPSADAPMFVMGVNHEK; VIHDNFGIVEGLMTTVHAITATQK; VPTPNVSVVDLTCR). These results suggested that the GluR2 subunit might form a protein complex with GAPDH through the GluR2<sub>NT</sub> domain. We then confirmed the GAPDH-GluR2 interaction with affinity purification experiments using GST-GluR2<sub>NT</sub>, GST-GluR2<sub>CT</sub> (I<sub>833</sub>-I<sub>883</sub>) and GST alone. Subsequent Western blot analysis using a GAPDH antibody confirmed the association between GluR2<sub>NT</sub> and GAPDH, but not GST-GluR2<sub>CT</sub> (Figure 4-1B). Thus, GAPDH interacted specifically with only the GluR2<sub>NT</sub>.

In order to confirm these results and delineate the region of the GluR2<sub>NT</sub> involved in the interaction with GAPDH, three GluR2<sub>NT</sub> GST-fusion proteins (GluR2<sub>NT1</sub>: V<sub>22</sub>-S<sub>271</sub>, GluR2<sub>NT2</sub>: K<sub>272</sub>-I<sub>421</sub>, GluR2<sub>NT3</sub>: L<sub>422</sub>-E<sub>545</sub>) were constructed (Figure 4-2G) and utilized in affinity purification experiments. As shown in Figure 4-2A, only GST-GluR2<sub>NT1</sub> precipitated GAPDH indicating that the GluR2 subunit interacted with GAPDH through its NT region V<sub>22</sub>-S<sub>271</sub>. A
Figure 4-1. Biochemical association of the GluR2 subunit with GAPDH.

A. Coomassie blue stained SDS-PAGE gel of the protein(s) selectively affinity pulled down by GST-GluR2NT, GluR1NT and GST alone from solubilized rat hippocampal lysates. Protein of interest: ~37kDa.

B. Western blot analysis of rat hippocampal proteins affinity purified by GST-GluR2NT, GST-GluR2CT and GST from solubilized rat hippocampal lysates and immunoblotted with GAPDH antibody.
series of truncations of the GluR2NT1 region were then created to map the site that interacts with GAPDH (Figure 4-2G). As shown in Figure 4-2 B and C, only GST-GluR2NT1-3 (H122-K172) and GST-GluR2NT1-3-2 (Y142-K172) were able to precipitate GAPDH from rat hippocampal tissue. While these results suggested the existence of an AMPAR/GAPDH protein complex, it did not clarify whether the AMPAR/GAPDH protein complex was formed through either a direct interaction or was mediated indirectly by other accessory binding proteins. To provide evidence that GAPDH and the GluR2 subunit directly interacted with each other we performed in vitro binding assays. As shown in Figure 4-2D, in vitro translated [35S]-GAPDH probe hybridized with GST-GluR2NT1 but not with GST-GluR2NT2, GST-GluR2NT3 or GST alone, indicating the specificity of the direct protein-protein interaction between GAPDH and GluR2NT1. Consistent with our affinity purification experiments, the in vitro translated [35S]-GAPDH probe only hybridized with GST-GluR2NT1-3 and GST-GluR2NT1-3-2, (Figure 4-2 E and F). Together, these data suggested that GAPDH formed a direct protein-protein interaction with the GluR2 subunit through the Y142-K172 region of the GluR2NT.

4.2.2 The complex formation between the endogenous GAPDH and GluR2 in situ is promoted by AMPAR activation and requires GAPDH-binding domain of GluR2NT1-3-2.

To provide evidence that the AMPAR: GAPDH complex could exist in vivo, we examined whether AMPAR and GAPDH could be co-immunoprecipitated from rat hippocampal tissue. As shown in Figure 4-3A, the GluR2 antibody was able to co-immunoprecipitate GAPDH from solubilized proteins extracted from the rat hippocampus. To investigate whether the GAPDH-GluR2 interaction occurred extracellularly or intracellularly, we performed cell surface biotinylation experiments in primary culture of rat hippocampus. Neurons were labeled with sulfo-NHS-LC-biotin. As shown in Figure 4-3B, the GluR2 antibody co-immunoprecipitated
Figure 4-2. Identification of the GluR2 subunit region involved in the GluR2-GAPDH interaction.

**A-C.** Rat hippocampal proteins affinity purified by (A) GST-GluR2NT1, GST-GluR2NT2 GST-GluR2NT3 and GST; (B) GST-GluR2NT1-1, GST-GluR2NT1-2, GST-GluR2NT1-3, GST-GluR2NT1-4, GST-GluR2NT1-5 and GST; (C) GST-GluR2NT1-3-1, GST-GluR2NT1-3-2 and GST from solubilized rat hippocampal lysates and immunoblotted with GAPDH antibody.

**D-F.** *In vitro* binding assay: [35S]-GAPDH probe bound with GST-GluR2NT1 (D), GSTGluR2NT1-3 (E) and GST-GluR2NT1-3-2 (F), but not with other GST fusion proteins or GST alone.

**G.** Schematic representation of GST-fusion proteins encoding truncated GluR2NT segments.
Figure 4-3. Secreted GAPDH interacts with the GluR2 subunit in the extracellular domain.

A. Co-immunoprecipitation of GAPDH by the GluR2 antibody from solubilized rat hippocampus.

B. Rat hippocampal neurons were incubated with sulfo-NHS-LC biotin to label cell surface proteins. GAPDH that co-immunoprecipitated with GluR2 antibody was examined in both non- and biotinylated (B) proteins.

C. Using a rabbit anti-GAPDH antibody, GAPDH was immunoprecipitated from the conditioned medium (CM; medium incubated with neurons/cells for 24 hours) of primary cultures of rat hippocampus but not from fresh medium. A mouse GAPDH antibody was used for Western blotting. Rabbit IgG: negative control.

D. GAPDH and α-tubulin in concentrated conditioned medium of non-transfected HEK-293T cells (non-T) and HEK-293T cells transfected with GluR1/2 subunits (AMPAR), in the presence/absence of glutamate (AMPAR+Glut). Cell lysates were used as controls.

E-F. Coimmunoprecipitation of GAPDH by GluR2 antibody (with/without glutamate treatment) from HEK-293T cells expressing GluR1/2 subunits (E) and hippocampal neurons (F) pre-treated with GluR2NT1-3-2 or GluR2NT-scram peptides (top panels). Each Coimmunoprecipitation was in parallel with Western blot analysis of the directly immunoprecipitated proteins (bottom panels).
GAPDH from the biotinylated (cell surface) fraction, but failed to co-immunoprecipitate GAPDH from the non-biotinylated (intracellular) fraction, suggesting that the AMPAR/GAPDH complex formation occurred extracellularly. Consistent with our findings, a recent study demonstrated that GAPDH was constitutively secreted into the extracellular space in several mammalian cell lines including HEK-293T cells and neuro-2a cells [48]. We therefore speculated that the secreted GAPDH may form a protein complex with GluR2NT. Thus, we first confirmed GAPDH secretion in our cell lines by immunoprecipitating GAPDH from the conditioned medium (incubation with neurons/cells for 24 hours) of hippocampal primary cultures with a primary antibody against GAPDH. As shown in Figure 4-3C, GAPDH was immunoprecipitated from serum-free conditioned medium, but not from serum-free fresh medium. To further exclude the possibility that the observed GAPDH released in the conditioned medium resulted from cell lysis, serum-free conditioned media from nontransfected HEK-293T cells and from cells expressing GluR1/2 subunits was collected, concentrated and examined by Western blot analyses using anti-GAPDH and anti-α-tubulin antibodies. As shown in Figure 4-3D, regardless of GluR1/2 subunit expression, GAPDH was detected from both conditioned media and cell lysates, whereas α-tubulin (a cytoplasmic protein marker) was only detected from cell lysates, indicating that the GAPDH found in the conditioned medium was secreted from cells and was not a contaminant due to cell lysis. Furthermore, we examined the effect of the AMPAR activation on the formation of AMPAR/GAPDH complexes. We found that AMPAR activation with either 100 µM glutamate in HEK-293T cells expressing GluR1/2 subunits or 100 µM KA in hippocampal neurons increased the co-immunoprecipitation of GAPDH with GluR2 by 75 ± 18% and 58 ± 11% (mean ± SEM, n=3), respectively (Figure 4-3 E, F; top panels). Agonist stimulation did not significantly alter the level of directly immunoprecipitated GluR2 subunit (Figure 4-3 E, F; bottom panels). Furthermore, if the GluR2NT1-3-2 region was important
for GluR2 to interact with GAPDH, application of the protein peptide encoding GluR2NT1-3-2 would be expected to disrupt the GAPDH/GluR2 interaction by competing with GluR2 for GAPDH. As expected, preincubation of the GluR2NT1-3-2 peptide (10 μM, 1 hour), not the scrambled GluR2NT1-3-2 peptide (GluR2NT1-Scram), significantly inhibited the agonist-induced increase in the AMPAR/GAPDH complex formation in transfected HEK-293T cells (Figure 4-3E, 65 ± 8% decrease; mean ± SE, n=3) and in hippocampal neurons (Figure 4-3F, 46 ± 6% decrease; mean ± SE, n=3. The fact that the extracellular application of the interfering GluR2NT1-3-2 peptide was able to disrupt the GAPDH/GluR2 interaction indicated that the GAPDH/GluR2 complex formation might occur extracellularly.

4.2.3 Disruption of GAPDH-GluR2 interaction inhibited AMPAR-mediated neuronal death in in-vitro models of excitotoxicity.

Both AMPAR and GAPDH have been independently shown to be involved in cell toxicity [49-53]. The observation that AMPAR activation promoted AMPAR/GAPDH complex formation suggested that the GAPDH-GluR2 protein-protein interaction might play a role in mediating AMPAR-mediated excitotoxicity. Consistent with previous studies [54, 55], treatment of HEK-293T cells expressing GluR1/2 with glutamate (300 μM, 24 hour; plus 25 μM cyclothiazide to prevent AMPAR desensitization) resulted in significant cell death (Figure 4-4A). Since the excessive influx of Ca^{2+} through glutamate receptors is thought to be responsible for the glutamate-induced excitotoxicity, we then examined the role of extracellular Ca^{2+} in the observed GluR2-mediated cell death. To test this possibility, HEK-293T cells expressing either GluR1/2 or NR1/2A were exposed to glutamate in the presence or absence of EGTA (5mM). As shown in Figure 4-4B, the NMDAR-mediated cell death was significantly reduced, while GluR1/2-mediated cell death remained intact in the presence of EGTA. This finding indicated
Figure 4-4. Regulation of the AMPAR-mediated cell death in HEK293T cells.

A. Quantitative measurements of PI fluorescence from HEK-293T cells expressing GluR1/2 subunits with/without glutamate treatment (300 μM glutamate, 25 μM CTZ, 24 hr).

***Significantly different from control group (n=9 per group, *P*<0.001); t-test.

B. PI fluorescence from HEK-293T cells expressing GluR1/2 or NR1/2A subunits treated with glutamate, in the presence or absence of EGTA (5 mM). *Significantly different from control group (n=9 per group, *P*<0.05); t-test.

C. PI fluorescence from HEK-293T cells expressing GluR1/2 subunits with/without glutamate treatment at various doses in the presence/absence of GluR2\_NT1-3-2 peptide (10 μM, 1 hr). **

*P*<0.01, ***P*<0.001; significantly different from control group. ANOVA, followed by *post-hoc* SNK test. ##, significant from the corresponding glutamate group (*P*<0.01, n=9 per group), t-test.

D. PI fluorescence from HEK-293T cells expressing GluR1/2 subunits with/without glutamate treatment in the presence/absence of GluR2\_NT1-3-2 peptide (10 μM, 1 hr). ***Significantly different from glutamate group (*P*<0.001; n=9 per group). ANOVA, followed by *post-hoc* SNK test.

E. Bar graph summarizing PI fluorescence from non-transfected HEK-293T cells or HEK-293T cells expressing GluR1/2 subunits. Cells were pre-treated with the GluR2\_NT1-3-2 peptide with/without glutamate treatment (n=9 per group).

F. PI fluorescence from HEK-293T cells expressing GluR1/3, GluR1/4 or GluR3/4 subunits with glutamate treatment in the presence/absence of the GluR2\_NT1-3-2 peptide (n=9 per group).
that the cell death induced by GluR2 AMPAR was not dependent on extracellular Ca\(^{2+}\) influx via the ionotropic receptor. To investigate the involvement of the GAPDH/GluR2 interaction in AMPAR-mediated cell death, HEK-293T cells expressing GluR1/2 were pre-treated with the GluR2\(_{NT1-3-2}\) peptide (10 μM, 1 hour). As shown in Figure 4-4C, pre-incubation with the GluR2\(_{NT1-3-2}\) peptide attenuated AMPAR-mediated cell death by 56 ± 2%, demonstrating that the disruption of the GAPDH-GluR2 coupling rescued cells from the AMPAR-mediated cell death. The GluR2\(_{NT1-3-2}\) peptide itself showed no effect in either the absence of glutamate treatment or in non-transfected cells regardless of glutamate treatment (Figure 4-4 C and D). The specificity of the GluR2\(_{NT1-3-2}\) peptide was also confirmed in HEK-293T cells expressing GluR1/3, GluR1/4 or GluR3/4 subunits, where pre-incubation with the GluR2\(_{NT1-3-2}\) peptide failed to inhibit AMPAR-mediated cell death (Figure 4-4E). Together, these data suggested that the GAPDH-GluR2 interaction played an important role in the GluR2 AMPAR-mediated cell death.

To examine the GAPDH-GluR2 interaction in a relevant cellular milieu, rat hippocampal neurons were utilized in parallel experiments. We previously demonstrated in Figure 4-3F that pre-incubating hippocampal neurons with the GluR2\(_{NT1-3-2}\) peptide disrupted the GAPDH/GluR2 interaction promoted by the AMPAR activation. Thus, we examined whether the disruption of this interaction in hippocampal neurons would rescue neurons from AMPAR-mediated excitotoxicity. AMPAR-mediated cell death was induced by treating neurons with KA (100 μM, 1 hour) in the presence of NMDAR and Ca\(^{2+}\) channel antagonists (10 μM MK-801 and 2 μM nimodipine, respectively). Pretreatment with the GluR2\(_{NT1-3-2}\) peptide significantly inhibited AMPAR-mediated cell death (Figure 4-5A), indicating that the disruption of AMPAR/GluR2 interactions leads to a significant decrease in AMPAR-mediated cell death in neurons. In order to further confirm the role of GAPDH in the AMPAR-mediated cell death, GAPDH siRNA (Santa Cruz Biotechnology, CA) was transfected into HEK-293T cells to block the expression of
**Figure 4-5. Regulation of the AMPAR-mediated cell death in rat hippocampal neurons.**

**A.** PI fluorescence from rat hippocampal primary culture with KA treatment (100 μM, 1hr) in the presence/absence of the GluR2_{NT1-3-2} peptide. ***Significantly different from KA group (n=9 per group, \( P<0.001 \)); t-test.

**B.** GAPDH in HEK-293T cells expressing GluR1/2 subunit in the presence/absence of GAPDH siRNA.

**C.** PI fluorescence from HEK-293T cells expressing GluR1/2 subunits with/without glutamate treatment in the presence/absence of GAPDH siRNA. *Significantly different from no GAPDH siRNA group (n=9 per group, \( P<0.05 \)); t-test. All PI fluorescence measurement assays and blots were performed 3 times independently.
GAPDH (Figure 4-5B). As shown in Figure 4-5C, AMPAR-mediated cell death was significantly attenuated in the presence of GAPDH siRNA. Together, these data further support that the GAPDH-GluR2 protein-protein interaction may contribute to the GluR2 AMPAR-mediated cell death.

4.2.4 Activation of AMPAR induces GAPDH internalization through GAPDH-GluR2 interaction

Previous studies demonstrated agonist stimulation induced AMPAR endocytosis [56-58]. Thus, we examined whether the extracellular GAPDH would internalize along with AMPAR through the GAPDH/GluR2 interaction upon the activation of AMPAR. Using a cell based ELISA assay previously described [58, 59] to quantify GluR2 and GAPDH cell surface levels in HEK-293T cells expressing GluR1/2, we found that the glutamate stimulation (100 μM, 30 minutes) induced a significant decrease in both plasma membrane GluR2 (Figure 4-6A) and in cell surface-associated GAPDH (Figure 4-6B). The ability of the GluR2NT1-3-2 peptide to abolish the glutamate-stimulated decrease in cell surface-associated GAPDH (Figure 4-6B), together with the inability of glutamate stimulation to internalize the cell surface-associated GAPDH in the absence of GluR1/2 subunits in HEK-293T cells, suggested that GAPDH internalization was a passive process enabled by the GAPDH/GluR2 interaction and was dependent on the GluR2 internalization (Figure 4-6C). The important role of the GluR2 subunit in cell surface-associated GAPDH internalization was also confirmed in HEK-293T cells expressing GluR1/3 subunits, where glutamate stimulation failed to induce the cell surface-associated GAPDH internalization (Figure 4-6D). Previous studies demonstrated that GluR2 endocytosis was dynamin-dependent and that the expression of the dominant-negative dynamin mutant (K44E) was able to block the GluR2 internalization [56, 58]. Thus, after confirming the ability of the K44E mutant to block
Figure 4-6. Activation of AMPAR induces GAPDH-GluR2 co-internalization.

A. GluR2 expression at the plasma membrane with/without glutamate treatment (100 μM, 30 minutes) in HEK-293T cells expressing GluR1/2 subunits. *Significantly different from control group (n=9 per group, $P<0.05$); t-test.

B. Cell surface-associated GAPDH with/without glutamate treatment in the presence/absence of the GluR2NT1-3-2 peptide in HEK-293T cells expressing GluR1/2 subunits. *Significantly different from control group; #, significantly different from glutamate group (n=9 per group, $P<0.05$); ANOVA, followed by post-hoc SNK test.

C. Cell surface-associated GAPDH in non-transfected HEK-293T cells with/without glutamate treatment (n=9 per group).

D. Cell surface-associated GAPDH with/without glutamate treatment in HEK-293T cells expressing GluR1/3 subunits (n=9 per group).
the GluR2 internalization (Figure 4-7A), we examined whether the K44E mutant affected cell surface-associated GAPDH internalization in HEK-293T cells expressing GluR1/2 subunits. As shown in Figure 4-7B, the K44E mutant significantly inhibited glutamate-induced cell surface-associated GAPDH internalization, indicating that GAPDH internalized through a dynamin-dependent pathway and further confirmed that GAPDH was co-internalize with the GluR2 subunit. Moreover, the K44E mutant also attenuated glutamate-induced cell death in HEK-293T cells expressing GluR1/2 subunits (Figure 4-7C), indicating that GAPDH/GluR2 complex internalization played a role in the GluR2 AMPAR-mediated cell death.

4.2.5 GAPDH and GluR2 translocate to the nucleus through the GAPDH-GluR2 interaction

Previous studies demonstrated that GAPDH initiated apoptotic cell death by nuclear translocation following Siah1 binding, and glutamate stimulation promoted GAPDH nuclear translocation [50, 60]. Therefore, we examined whether the internalized GAPDH was translocated to the nucleus upon AMPAR agonist stimulation. Surprisingly, not only GAPDH but also GluR2 exhibited a significant increase in nuclear localization following the activation of AMPAR in either HEK-293T cells expressing GluR1/2 subunits (Figure 4-8 A-C) or in rat hippocampal neurons (Figure 4-8 D-F). Furthermore, the nuclear translocation of GAPDH and GluR2 was blocked by pre-incubation with GluR2NT1-3-2 peptide (Figure 4-8 A-F). LaminB1 was used as the nuclear marker, and α-tubulin was used as the cytoplasmic marker to demonstrate that the nuclear fraction was relatively free of intracellular organelles. To confirm whether the observed nuclear GAPDH and GluR2 originated from the cell surface, hippocampal neurons were first labeled with sulfo-NHS-SS-Biotin (cell permeable) and then treated either with or without the GluR2NT1-3-2 peptide prior to agonist stimulation. Subsequently, all cell surface biotin
Figure 4-7. Dynamin is involved in the glutamate-induced GAPDH-GluR2 cointernalization.

**A-B.** Quantification of plasma membrane GluR2 (A) or cell surface-associated GAPDH (B) expression at the plasma membrane with/without glutamate treatment in HEK-293T cells expressing GluR1/2 subunits with wild type dynamin (WT) or mutant K44E dynamin (K44E). *Significantly different from the corresponding control group (n=9 per group, \(P<0.05\)); t-test.

**C.** PI fluorescence from HEK-293T cells expressing GluR1/2 subunits with wild type dynamin (WT) or mutant K44E dynamin (K44E) with/without glutamate treatment. ***Significantly different from control WT group (n=9 per group, \(P<0.001\)); ##significantly different from control K44E group (n=9 per group, \(P<0.01\)); t-test. All assays in this figure were performed 3 times independently.
**Figure 4-8. Activation of AMPAR induces GAPDH-GluR2 nuclear translocation.**

*A*, GluR2 and GAPDH expression in the nucleus with/without glutamate treatment in the presence/absence of the GluR2NT1-3-2 peptide in HEK-293T cells expressing GluR1/2 subunits. LaminB1 and α-tubulin were used as the nuclear and cytoplasmic marker, respectively. Whole cell lysates were used as a control.

*B-C*, Quantification of nuclear expression of GluR2 (**B**) and GAPDH (**C**). **P<0.01, * P<0.05; significantly different from control group (n=3 per group). ANOVA, followed by post-hoc SNK test.

*D*, GluR2 and GAPDH expression in the nucleus with/without KA treatment in the presence/absence of GluR2NT1-3-2 peptide in rat hippocampal primary culture. LaminB1 and α-tubulin were used as nuclear and cytoplasmic marker, respectively. Whole cell lysates were used as a control.

*E-F*, Quantification of nuclear expression of GluR2 (**E**) and GAPDH (**F**). The intensity of each protein bands was quantified by densitometry (software: ImageJ, Bethesda, MD). *** P<0.001, ** P<0.01; significantly different from control group (n=3 per group). ANOVA, followed by post-hoc SNK test.
was cleaved by using GSH (glutathione), leaving only the endocytosed proteins labeled with biotin. The ability of GSH to cleave all cell surface biotin was confirmed (Figure 4-9A). Then nuclear proteins were extracted followed by the biotin purification using streptavidin beads, in order to harvest biotinylated nuclear proteins. As shown in Figure 4-9B, Western blots from SDS-PAGE of nuclear extracts that were streptavidin purified revealed that the levels of biotinylated GAPDH and GluR2 was significantly increased in the nuclear extract of hippocampal neurons upon the agonist stimulation, a phenomenon that was blocked by the preincubation with the GluR2$_{NT1-3-2}$ peptide. In an attempt to directly visualize the translocation of cell surface GluR2 and cell surface-associated GAPDH to the nucleus, we used immunofluorescent confocal microscopy to detect the re-distribution of cell surface GluR2 and cell surface-associated GAPDH following glutamate treatment in HEK-293T cells expressing GluR1 with HA-GluR2 subunits. We first looked at the nuclear translocation of GluR2. Cell surface HA-GluR2 was labeled with anti-HA antibody in living cells. After glutamate stimulation, cells were fixed and stained with Cy2 conjugated secondary antibody (green) under non-permeabilized conditions (for cell surface GluR2) and then Cy3 conjugated secondary antibody (red) under permeabilized conditions (for internalized GluR2). As shown in Figure 4-10A, GluR2 localizes at cell surface only in the absence of agonist stimulation. Following glutamate stimulation, internalized GluR2 was detected in the nucleus. We then examined the nuclear translocation of extracellular GAPDH. Commercially available GAPDH was labeled with HiLyteFluo555 and added into cell culture medium. Cells expressing GluR1 with HA-GluR2 subunits were treated with glutamate, fixed and stained with anti-HA antibody. As shown in Figure 4-10B, the extracellular HiLyteFluo555-GAPDH (red) was clearly seen inside the cells and localized in the nucleus only in cells expressing GluR2 (green) following glutamate treatment. By combining the two strategies mentioned above, we were able to detect the co-
Figure 4-9. Nuclear GAPDH and GluR2 originated from the cell surface.

A. Western blot analysis of biotin in HEK-293T cells. After incubation with sulfo-NHS-SS–biotin (cell permeable), cells were kept at 4°C to block basal endocytosis and then treated with/without glutathione. Cells were then homogenized and subjected to SDS-PAGE.

B. Biotinylated nuclear GluR2 (top) and GAPDH (bottom) obtained from hippocampal neurons. Neurons were labeled with sulfo-NHS-SS-biotin and then treated with or without the GluR2NT1-3-2 peptide before KA treatment.
Figure 4-10. Confocal microscopy of the GAPDH-GluR2 nuclear translocation.

A. Confocal microscopy of HEK-293T cells expressing HA-tagged GluR2 (HA-GluR2) and GluR1 subunits with/without glutamate treatment. Cells exhibit green fluorescence indicating HA-GluR2 subunit on plasma membrane (the 1st row) while the internalized HA-GluR2 subunits exert red fluorescence (the 2nd row). Nuclei were stained with Topro3 blue (the 3rd row) and the last row shows the merge image. Bar=10 μm.

B. Confocal microscopy of HEK-293T cells expressing GluR1/HA-GluR2 subunits with/without glutamate treatment in the presence of the HiLyteFluo555 labeled-GAPDH. Cells exhibit green fluorescence indicating HA-GluR2 subunit (the 1st row) while the HiLyteFluo555 labeled-GAPDH exert red fluorescence (the 2nd row). Nuclei were stained with Topro3 blue (the 3rd row) and the last row shows the merge image. Bar=10 μm.

C. Confocal microscopy of HEK-293T cells expressing GluR1/HA-GluR2 subunits treated with glutamate in the presence of the HiLyteFluo555-labeled GAPDH. Cells exhibit green fluorescence indicating HA-GluR2 subunit while the HiLyteFluo555 labeled-GAPDH exerts red fluorescence. The merge images show the co-localization of HA-GluR2 and GAPDH. Bar=10 μm.
translocation of the cell surface GluR2 and the extracellular HiLyteFluo555-GAPDH following glutamate treatment (Figure 4-10C). These data further support that GluR2 and GAPDH co-translocation to nucleus following agonist stimulation of AMPAR.

4.2.6 Activation of AMPAR facilitates nuclear GAPDH-p53 coupling

p53, a tumor suppressor and transcription factor, which can also initiate apoptosis, was previously implicated in the glutamate-mediated excitotoxicity [61-63]. More interestingly, a previous study showed an interaction between GAPDH and p53 involved in neuronal apoptosis [64]. Thus, we tested whether GluR2NT and GAPDH would interact with p53 using affinity “pull down” experiments. Interestingly, only GST-GAPDH, but not GST-GluR2NT or GST alone, affinity precipitated p53 from nuclear extracts of rat hippocampal neurons (Figure 4-11A). In addition, as shown in Figure 4-11B, GAPDH co-immunoprecipitated with p53 from isolated nuclei of HEK-293T cells expressing GluR1/2 subunits indicating that GAPDH and p53 form a protein complex that appeared to be facilitated by AMPAR activation. We also found that p53 acted as a competitive inhibitor to the GAPDH-GluR2 coupling as the pretreatment with the GluR2NT1-3-2 peptide, but not the scrambled GluR2NT1-3-2 peptide, inhibited GAPDH/p53 interactions (Figure 4-11B). Furthermore, pre-incubation with purified GST-p53, but not GST alone, inhibited the GAPDH/GluR2 coupling in a concentration dependent manner, as indexed by affinity “pull down” experiments (Figure 4-11C). To identify the p53 interacting domain on GAPDH, GST-fusion proteins encoding fragments of GAPDH were constructed and used in affinity purification assays (Figure 4-12A). The results revealed that the sequence encoding GAPDH: I_{221}-E_{250} facilitated the interaction with p53, since only GST-GAPDH_{2-2-1-1} (I_{221}-E_{250}) was able to pull down p53 from solubilized nuclear protein extracts derived from rat hippocampus (Figure 4-12 B-E). Furthermore, we confirmed the important role of the I_{221}-E_{250}
Figure 4-11. Activation of AMPAR facilitates nuclear GAPDH-p53 interaction.

A. Nuclear p53 in hippocampus after affinity precipitation by GST-GAPDH, GSTGluR2NT or GST alone.

B. Co-immunoprecipitation of GAPDH with p53 from extracted nuclear proteins of HEK-293T cells expressing GluR1/2 subunits pre-treated with/without GluR2NT1-3-2 peptide or GluR2NT-scram peptide followed by glutamate treatment.

C. Western blot of nuclear GAPDH in HEK-293T cells expressing GluR1/2 subunits after affinity precipitated by GST-GluR2NT in the presence of increasing concentration of GST-p53 or GST.
Figure 4-12. Identification of the GAPDH region involved in the GAPDH-p53 interaction.

A. Schematic representation of GST-fusion proteins encoding truncated GAPDH segments.

B-E. Nuclear p53 in hippocampus after affinity precipitation by GST-GAPDH1 and GST-GAPDH2 (B), GST-GAPDH2-1 and GST-GAPDH2-2 (C), GST-GAPDH2-2-1 and GSTGAPDH2-2-2 (D), GAPDH2-2-1-1, GAPDH2-2-1-2 and GAPDH2-2-1-3 (E).

F. Co-immunoprecipitation of nuclear p53 by GAPDH primary antibody in HEK-293T cells co-expressing GluR1/2 subunits with GAPDH2-2-1-1 mini-gene.
region in maintaining the GAPDH-p53 coupling. As shown in Figure 4-12F, co-expression of a mini-gene encoding the GAPDH$_{2,2,1,1}$ region blocked the co-immunoprecipitation of p53 with GAPDH. Similarly, we conducted a series of affinity “pull down” experiments to identify the GluR2 interaction domain on GAPDH. As shown in Figure 4-13 A-D, GluR2 was pulled down specifically by GST-GAPDH$_{2,2,1,1}$ indicating that GluR2 and p53 may share the same binding site on GAPDH. These data further confirmed that GluR2 and p53 bind to GAPDH in a competitive manner.

4.2.7 GAPDH-p53 plays an important role in GluR2 AMPAR-mediated cell death

Both GAPDH and p53 have been independently shown to be involved in cell toxicity [49, 62]. We suspected that the sequential protein-protein interactions among GluR2, GAPDH and p53 and nuclear translocation events might play an important role in mediating AMPAR-mediated excitotoxicity. To investigate the role of p53 in the GluR2 AMPAR-mediated neurotoxicity, we pre-treated HEK-293T cells expressing GluR1/2 subunits with the p53 inhibitor PFT-α (pifithrin-α, 10 μM, 1 hour) prior to glutamate treatment. PFT-α is a p53 inhibitor that was previously used to protect neurons against apoptosis induced by DNA-damaging agents, amyloid β-peptide, glutamate and ischemia (51, 52). As shown in Figure 4-14A, PFT-α treatment significantly inhibited glutamate-induced cell death in HEK-293T cells expressing GluR1/2 subunits, but failed to inhibit glutamate-induced cell death in HEK-293T cells expressing GluR1/3 subunits suggesting that the GluR2 AMPAR-mediated cell death was p53-dependent (Figure 4-14B). To examine whether the GAPDH-p53 coupling played a functional role in GluR2 AMPAR-mediated cell death we co-transfected the GAPDH$_{2,2,1,1}$ mini-gene in HEK-293T cells expressing GluR1/2. Expression of the mini-gene resulted in the
Figure 4-13. Identification of the GAPDH region involved in the GAPDH-GluR2 Interaction.

**A-D.** Hippocampal GluR2 after affinity precipitation by GST-GAPDH₁ and GSTGAPDH₂ (A), GST-GAPDH₂-₁ and GST-GAPDH₂-₂ (B), GST- GAPDH₂-₂-₁ and GSTGAPDH₂-₂-₂ (C), GAPDH₂-₂-₁-₁, GAPDH₂-₂-₁-₂ and GAPDH₂-₂-₁-₃ (D).
Figure 4-14. GAPDH-p53 plays an important role in GluR2 AMPAR-mediated cell death.

A. Glutamate-induced cell death data in the presence/absence of p53 antagonist cyclic PFT-α (10 μM, 1hr) in HEK-293T cells expressing GluR1/2 subunits through PI fluorescence.

*Significantly different from control group ($P<0.05$; $n=9$ per group); t-test.

B. Glutamate-induced cell death data in the presence/absence of p53 antagonist cyclic PFT-α in HEK-293T cells expressing GluR1/3 subunits through PI fluorescence ($n=9$ per group).

C. Glutamate-induced cell death data obtained from HEK-293T cells co-expressing GluR1/2 subunits with/without the GAPDH2-2-1-1 mini-gene. **Significantly different from non treatment/transfection group ($P<0.01$; $n=9$ per group); ANOVA, followed by post-hoc SNK test.

D. Nuclear p53 and p53 phosphorylation at serine 46 in HEK-293T cells co-expressing GluR1/2 subunits with the GAPDH2-2-1-1 mini-gene upon glutamate stimulation.
disruption of GAPDH/p53 interactions, as previously shown in co-immunoprecipitation experiments (Figure 4-12F). As shown in Figure 4-14C, the GluR2 AMPAR-mediated cell death was also significantly inhibited in HEK-293T cells co-expressing the GAPDH2-2-1-1 mini-gene indicating the critical role of the GAPDH/p53 coupling in the GluR2 AMPAR-mediated cell death. Previous studies demonstrated a strong correlation between p53 expression and excitotoxic neuronal death [61-63], while other studies reported phosphorylation can regulate p53 activity [65-69]. Thus, we tested whether enhancing the GAPDH/p53 coupling in response to AMPAR activation affected p53 expression and/or phosphorylation. As shown in Figure 4-14D, activation of AMPAR resulted in a 143±16% (mean±SE, n=3, p<0.05) increase in the p53 expression and a 147±6 % (mean±SE, n=3, p<0.05) increase in the p53 phosphorylation in HEK-293T cells expressing GluR1/2 subunits, but this was not observed in the presence of the GAPDH2-2-1-1 mini-gene. Together, these data suggested that the GAPDH-p53 protein-protein interaction also played a critical role in GluR2 AMPAR-mediated cell death.

4.2.8 Application of GluR2NT1-3-2 peptide protects brain damage from global ischemia in the rat hippocampus

We hypothesized that the GAPDH-GluR2 complex-initiated cell death pathway played a critical role in the ischemic brain damage. To test this possibility, we first examined the effect of transient global cerebral ischemia (10 minutes occlusion of common carotid arteries) on AMPAR/GAPDH and GAPDH/p53 complex formation, as well as agonist-stimulated nuclear translocation of both GluR2 and GAPDH. To facilitate the intracellular delivery of the peptide, both the GluR2NT1-3-2 peptide and scrambled GluR2NT1-3-2 peptide were fused to the cell membrane transduction domain of the HIV-1 TAT protein [YGRKKRRQRRR [70]] to generate TAT-GluR2NT1-3-2 and TAT-GluR2NT-Scram as previously described [71]. Although we demonstrated that GAPDH and GluR2 form complex extracellularly, we utilized the TAT-tagged
peptide to block both extracellular and intracellular GAPDH/GluR2 complex formation. The protective effect of TAT-GluR2\textsubscript{NT1-3-2} peptide was first confirmed in HEK-293T cells expressing GluR1/2 subunits, in parallel with GluR2\textsubscript{NT1-3-2} peptide (Figure 4-15A). Consistent with our hypothesis, co-immunoprecipitation of GAPDH with GluR2 subunit from sham, ischemia and the TAT-GluR2\textsubscript{NT1-3-2} peptide-treated rat brains revealed an increase in the GAPDH/GluR2 interaction in ischemia rats, an effect that was inhibited by the TAT-GluR2\textsubscript{NT1-3-2} peptide (0.5 μl, 10 mM) administrated 2 hours after ischemia via stereotaxic hippocampal injection (Figure 4-15 B, C). Furthermore, we also observed a similar change in the GAPDH/p53 interaction in the nuclear extracts (Figure 4-15 D, E). The nuclear translocation of both GluR2 and GAPDH in ischemia rat brains was also inhibited by the application of the TAT-GluR2\textsubscript{NT1-3-2} peptide (Figure 4-16 A-C). We then assessed the ability of the TAT-GluR2\textsubscript{NT1-3-2} peptide to mitigate neuronal cell death in rats subjected to transient global cerebral ischemia. Ischemia-induced neuronal death of pyramidal cells in the CA1 subfield (Figure 4-17A) was elucidated by cresyl violet staining (Figure 4-17 B-G). In the hippocampus, sham-operated rats showed no neuronal damage (Figure 4-17 B, C), while rats subjected to ischemia showed significant neuronal loss in the hippocampus 5 days after ischemia, with only ~19% of hippocampal CA1 pyramidal neurons surviving compared to the CA1 from sham-operated rats (Figure 4-17 D, E, H). Stereotaxic injection of 5 nmol TAT-GluR2\textsubscript{NT1-3-2} peptide into the hippocampus 2 hours after ischemia significantly improved neuronal survival in the hippocampal CA1 region 5 days after ischemia (Figures 4-17 F, G, H; post-ischemia: 47.4 ± 7.9%; pre-ischemia: 58.9 ± 7.6%). Injection of the TAT-GluR2\textsubscript{NT1-3-2} peptide 1 hour before ischemia also rescued CA1 pyramidal cells, whereas the scrambled peptide injection (post-ischemia) did not have any significant effects (Figure 4-17H). Thus, the GAPDH/GluR2 interaction may be involved in ischemia-induced cell loss and the
Figure 4-15. Effect of transient global cerebral ischemia on GAPDH-GluR2 and GAPDH-p53 complex formation.

A. Bar graph summarizing the cell death data obtained from HEK-293T cells expressing GluR1/2 subunits in the presence of glutamate treatment. HEK-293T cells were pretreatment with TAT-GluR2NT1-3-2 peptide, non-TAT-linked GluR2NT1-3-2 peptide or scramble of GluR2NT1-3-2 peptide. ** $P<0.01$, *** $P<0.001$; significantly different from glutamate group (n=9 per group); ANOVA, followed by post-hoc SNK test.

B. Coimmunoprecipitation of GAPDH by GluR2 antibody from rat hippocampal extracts of sham, peptide-treated (post-ischemia) and ischemia groups.

C. Quantification of GAPDH-GluR2 coimmunoprecipitation from sham, peptide-treated (post-ischemia) and ischemia groups. *Significantly different from control group ($P<0.05$); #significantly different from ischemia group (n=3 per group, $P<0.05$). ANOVA, followed by post-hoc SNK test.

D. Coimmunoprecipitation of GAPDH by p53 from nuclear proteins extracted from rat hippocampus of sham, peptide-treated (post-ischemia) and ischemia groups.

E. Quantification of the GAPDH-p53 coimmunoprecipitation from sham, peptide-treated (post-ischemia) and ischemia groups. *Significantly different from control group (n=3 per group, $P<0.05$). ANOVA, followed by post-hoc SNK test.
Figure 4-16. Effect of transient global cerebral ischemia on GAPDH-GluR2 nuclear translocation.

_A._ GAPDH and GluR2 nuclear expression in rat hippocampal tissues from sham, peptide treated (post-ischemia) and ischemia groups.

_B-C._ Quantification of GluR2 (B) and GAPDH (C) nuclear expression in rat hippocampal tissues from sham, peptide-treated (post-ischemia) and ischemia groups. **Significantly different from peptide group (n=3 per group, P<0.01). Due to the low GluR2 levels in the control group, data were normalized to peptide group; t-test (B). For (C), **significantly different from sham group (P<0.01). ##significantly different from ischemia group (n=3 per group, P<0.01); ANOVA, followed by post-hoc SNK test.
Figure 4-17. Protective effects of the TAT-GluR2NT1-3-2 peptide in animal model of global ischemia.

A. Schematic diagram of hippocampus.

B-G. Representative images of rat hippocampus and hippocampal CA1 subfield stained with cresyl violet in sham (B-C), ischemia (D-E) or treated with 5 nmol peptide 2 hrs after ischemia (F-G).

H. Bar plot summarizing the relative pyramidal cell survival in the hippocampal CA1 subfield from Sham, peptide (Pep) 1 hour before (pre) or 2 hours after (post) ischemia and TAT-scrambled peptide (Scram) 2 hours after (post) ischemia. Bars show the relative mean ± SE of living cells stained by cresyl violet. ***Significantly different from sham group (P<0.001); ## P<0.01; ### P<0.001; significantly different from ischemia group (n=6 per group). ANOVA, followed by post-hoc SNK test.
SO, stratum oriens; SP, stratum pyramidale; SR, stratum radiatum.
TAT-GluR2\textsubscript{NT1-3-2} peptide, which can disrupt the GAPDH-GluR2 interaction \textit{in vivo}, was capable of protecting neurons from the global ischemia-induced cell death.

4.2.9 GluR2\textsubscript{NT1-3-2} peptide protects brain damage from focal ischemia in rat

We then examined whether the pre-treatment with the TAT-GluR2\textsubscript{NT1-3-2} peptide would reduce focal ischemia-induced brain damage. Adult male Sprague-Dawley rats were subjected to MCAO (transient middle cerebral artery occlusion) for 90 minutes by the intraluminal suture method [72, 73]. Animals were treated with a single stereotaxic ICV injection with 40 nmol TAT-GluR2\textsubscript{NT1-3-2} peptide 60 minutes before or 30 minutes after MCAO. Body temperature, blood pressure, and blood gases were monitored and maintained throughout the experiment (data not shown). The extent of the cerebral infarction was measured 24 hours after MCAO onset. The postural reflex test [72] and the forelimb placing test [73] were used to grade neurological function on a scale of 0 to 12 (normal = 0; worst = 12) 2 hours and 24 hours after MCAO. As shown in Figure 4-18 A-C, animals treated with the TAT-GluR2\textsubscript{NT1-3-2} peptide before and after MCAO and GluR2\textsubscript{NT1-3-2} peptide after MCAO exhibited a significant reduction in the volume of total cerebral infarction by 82 ± 3%, 34 ± 11% and 28 ± 9% respectively, compared with untreated MCAO lesion animals, respectively (mean \pm SE, n=9, \( p<0.01 \)). Furthermore, animals treated with either the TAT-GluR2\textsubscript{NT1-3-2} peptide or GluR2\textsubscript{NT1-3-2} peptide before and after MCAO also displayed a significant improvement in 24-hour neurological scores compared with that at 2 hours (Figure 4-18D; mean \pm SE, n=9, \( p<0.001 \)). Taken together, our data demonstrate that the GAPDH-GluR2 interaction interfering peptide was capable of protecting neurons from both global ischemia- and focal ischemia-induced cell death.
**Figure 4-18.** TAT-GluR2NT1-3-2 peptide promoted neuronal survival in *in vivo* model of tMCAO.

**A.** Representative images of rat brain sections stained with TTC from rats subjected to tMCAO and the effects of 40 nmol peptide (Pep) 1 hour before (pre) or 1/2 hour after (post) ischemia onset via ICV injection. Non-TAT-linked peptide (40 nmol) was injected 1/2 hour after (post) ischemia. Each group has 9 animals except for the ischemia group that has 7 animals. White area: cerebral infarction.

**B-C.** Quantification of infarct area and infarct volume of rat brain sections stained with TTC in ischemia, the scramble peptide administrated after ischemia (Scram), the peptide administrated before (Pre)/after ischemia (post) and the non-TAT-linked GluR2NT1-3-2 peptide administrated after ischemia. *Significantly different from the ischemia group (P<0.01). ANOVA, followed by post-hoc SNK test.

**D.** Neurological deficit scores (0, normal; 12, worst) assessed 2 hours and 24 hours after ischemia in ischemia, scramble peptide administrated after ischemia (Scram), the peptide administrated before (Pre)/after ischemia (post) and the non-TAT-linked GluR2NT1-3-2 peptide administrated after ischemia. *Significantly different from the corresponding 2 hrs group, P<0.01, t-test.
4.3 Discussion

AMPAR-mediated excitotoxicity has been implicated in the pathogenesis of neuronal loss associated with a number of brain disorders, including transient forebrain ischemia [21-33]. However, the underlying mechanisms remain unclear. An uncontrolled rise in intracellular Ca\(^{2+}\) and Zn\(^{2+}\) and the subsequent activation of diverse downstream cell death signals have been considered one of the most prominent hypotheses to explain excitotoxic neuronal death [32, 33, 74-78]. Recent studies have suggested that increased numbers of GluR2-lacking AMPARs are present after ischemic injury, which may be the consequence of GluR2 internalization which results in decreased GluR2 expression at the cell surfaces [29, 79-82]. These changes have been associated with an increased vulnerability of hippocampal pyramidal neurons to ischemic injury [83]. They are also consistent with our observation that agonist-induced GAPDH/GluR2 formation results in the internalization of the GluR2 subunits and leads to the subsequent activation of p53-dependent cell death pathway. Moreover, we have found that chelating extracellular Ca\(^{2+}\) with EGTA to block NMDAR-mediated excitotoxicity has little effect on the GluR2 AMPAR-mediated cell death. This result suggests that under our experimental conditions GluR2 AMPAR-mediated excitotoxicity is not mediated by Ca\(^{2+}\) influx, but is instead mediated by a previously unappreciated mechanism. We have been able to demonstrate for the first time that the GluR2 AMPAR-mediated neurotoxicity is mediated by a novel mechanism that involve several steps: (1) secreted GAPDH forms a protein complex with AMPAR at the extracellular cell surface through its direct interaction with the GluR2\textsubscript{NT}, (2) AMPAR activation promotes co-internalization and subsequent nuclear translocation of GluR2 and GAPDH, and (3) the formation of the nuclear GAPDH/p53 complex and the activation of nuclear p53-mediated cell death pathways. Through these multiple sequential steps, extracellular GAPDH translates the activation of plasma membrane AMPAR to the activation of p53-dependent cell death signaling.
pathway in the nucleus resulting in excitotoxic neuronal death. Moreover, we have shown that administration of the interfering GluR2NT1-3-2 peptide significantly mitigates the ischemia-induced neuronal cell death in animal models of global ischemia and focal ischemia.

The development of therapeutic proteins that has presented a valuable method to treat diseases is limited by low efficiency of traditional delivery methods. Recently, several groups have successfully delivered TAT-fused proteins \textit{in vitro}. TAT was able to deliver different proteins, such as horseradish peroxidase and RNAse A across cell membrane into the cytoplasm in different cell lines \textit{in vitro}. But the size range of proteins with effective delivery is restricted from 30kDa to 120-150kDa. In one study, TAT fused proteins are rapidly internalized by lipid raft-dependent macropinocytosis using a transducible TAT–Cre recombinase reporter assay on live cells [113]. In another study, Tat-fusion protein was delivered into mitochondria of breast cancer cells and decreased the survival of breast cancer cells, which showed capability of TAT-fusion proteins to modulate mitochondrial function and cell survival. However, the \textit{in vivo} application of TAT-fused proteins is limited by a lack of cell specificity in TAT-mediated cargo delivery and insufficient understanding of the modes of their uptake [114].

Furthermore, non-covalent method that forms TAT/protein complexes has also been developed to address the limitations in covalent method such as chemical modification before cross linking and denaturation of proteins before delivery.

It should also be noted that the TAT is the trans-activating transcriptional activator from Human Immunodeficiency Virus 1, and similar to other exogenous antigens, TAT-fused proteins may lead to immune responses.

It is somewhat surprising to find that the extracellular GluR2_{NT} can interact and form a protein complex with GAPDH, a key enzyme involved in glycolysis and thought to be
ubiquitously distributed intracellularly. However, additional roles for GAPDH have recently been reported including involvement in membrane fusion/transport, binding to low molecular weight G proteins, regulation of the cytoskeleton, the accumulation of glutamate into presynaptic vesicles and apoptosis [84-90]. Furthermore, there is evidence indicating that GAPDH secretion in mammalian cells plays a role in cell adhesion [48, 91-93]. Consistent with previous reports, our data confirm the presence of GAPDH in the extracellular space. Biotinylation data, together with the ability of the extracellular GluR2NT1-3-2 peptide to disrupt the GAPDH/GluR2 coupling provide evidence that the GAPDH/GluR2 interaction occurs at the cell surface.

Reduction in the plasma membrane expression of GluR2 is associated with the AMPAR-mediated cell death [80, 94]. However, the mechanism(s) involved is not clear, although it has been suggested that the GluR2 internalization may enhance Ca^{2+}-influx that subsequently results in neurotoxicity either through newly synthesized Ca^{2+}-permeable AMPARs [80] or by activation of a caspase dependent apoptotic pathway [95]. Consistent with previous studies, our data has shown that agonist stimulation of AMPAR results in the internalization of GluR2 and promotes extracellular GAPDH internalization via a process dependent on the GAPDH-GluR2 coupling. Many studies have shown that the agonist-induced GluR2 internalization is a dynamin dependent process [56, 58]. The fact that mutant dynamin abolishes both GluR2 and GAPDH internalization and the inability of GAPDH to internalize in cells lacking GluR2 expression suggests that GAPDH internalization is a passive process facilitated by GAPDH/GluR2 interactions and is mediated by GluR2 internalization. Furthermore, GAPDH internalization is dependent on the GluR2 subunit, since there is an absence of GAPDH internalization in nontransfected cells or in HEK-293T cells coexpressing GluR1/3 subunits treated with glutamate.
In investigating possible mechanisms that may underlie this GluR2/GAPDH related cell death, we have found that glutamate treatment increases both p53 phosphorylation and protein levels. A large body of work indicates that almost any DNA-damaging agent can cause the apoptosis of neurons, including sympathetic, cortical, hippocampal and cerebellar neurons, and that in all cases this apoptosis is dependent upon p53 [96]. The first indication that p53 might be important for neuronal damage following ischemia or excitotoxicity came from studies showing that p53 levels increase in response to these insults [96]. Of particular interest are studies demonstrating (i) that neuronal death in the hippocampal CA1 region was much more extensive in p53+/+ than in p53−/− mice subjected to transient global ischemia by the three-vessel occlusion method [97], and (ii) that transient focal ischemia induced by MCAO led to significant ischemic damage in p53+/− but not in p53+/+ mice [98]. The molecular mechanisms by which p53 is activated and accumulates under conditions of cellular stress may include either phosphorylation or acetylation of p53 [99]. In the present study, the p53 inhibitor PFT-α treatment significantly prevents glutamate-induced cell death in HEK-293T cells expressing GluR1/2 subunits, suggesting the involvement of p53 in this AMPAR-mediated cell death. Moreover, p53 directly binds to GAPDH and the disruption of this protein-protein interaction significantly inhibits glutamate-induced cell death and nuclear p53 accumulation/phosphorylation. Interestingly, the GAPDH-GluR2 interaction competes with the GAPDH/p53 coupling since the recombinant p53 protein is able to inhibit GluR2/GAPDH interactions in a concentration dependent manner. Thus, the formation of the GAPDH/p53 complex can then activate p53-dependent cell death signals leading to neuronal death.

Stroke is the second leading cause of death worldwide yet there are very few effective pharmacological options for individuals suffering an ischemic stroke. Thrombolytics, such as alteplase and tenecteplase have been a significant advance in the treatment of ischemic stroke.
However, the efficacy of thrombolytics necessitates early intervention (within 3 hours of ischemic episode). This has limited their practical use in much of the world. There continues to be a significant unmet need for acute pharmacological treatments that go beyond the use of thrombolytics. Advances in recent years include: hypothermia [100-102], oxygen therapy [103], stem cells transplantation [104] and cerebral plasticity stimulation (trophic factors) strategies [105]. These novel techniques are intriguing, but will require further well-designed prospective trials to assess clinical feasibility, safety, and efficacy [106]. Another area that has received considerable attention has been the development and clinical testing of agents that attempted to inhibit ischemia-induced excitotoxicity by directly using glutamate receptor antagonists. However, although many promising preclinical and clinical agents have been investigated, all have failed at various stages of development for a variety of reasons. One of the key issues accounted for the failure is the fact that glutamate receptor antagonists are able to block excitatory neurotransmission that is necessary for maintaining normal brain function. Thus, our results reveal a previously unappreciated signaling pathway underlying AMPAR-mediated excitotoxicity and may provide a new avenue for the development of a complementary therapeutics in the treatment of neuropathological disorders, such as stroke and epilepsy.
4.4 Materials and Methods

Primary hippocampal neuron culture, GST fusion proteins and mini-genome, protein affinity purification, in vitro binding, co-immunoprecipitation and Western blot, Cell-ELISA assays were performed as previously described in our group [107-109]. The details of the experimental procedures can be found in the supplemental data.

4.4.1 Quantification of AMPAR-mediated Excitotoxicity

HEK-293T cells transfected with GluR1/2 subunits were exposed to 300 µM glutamate/25 µM cyclothiazide at 37°C for 24 hour. Cells were allowed to recover for 24 hours at 37°C. To quantify AMPAR-mediated cell death, culture medium was replaced by extracellular solution containing 50 µg/ml of propidium iodide (PI) (Invitrogen, Carlsbad, CA). After 30 minutes incubation at 37°C, fluorescence intensity in each well was measured with a plate reader (Victor3; PerkinElmer, Waltham, MA). The fraction of dead cells was normalized to the cell toxicity that occurred in either the glutamate-treated cells or KA-treated neurons. Primary hippocampal neurons were exposed to 100 µM KA/25 µM cyclothiazide in the presence of NMDAR and Ca2+ channel antagonists (10 µM MK-801 and 2 µM nimodipine, respectively) at 37°C for 1 hour.

4.4.2 Cell Biocytination

For cell surface biocytination, cells were rinsed four times with ice-cold PBS containing 0.1 mM CaCl2 and 1.0 mM MgCl2 (PBS2+) after treatment, and incubated twice with 1.0 mg/ml sulfo-NHS-LC-biotin (Pierce, Rockford, IL) for 20 minutes at 4°C. Non-reactive biotin was quenched by 20 minutes incubation at 4°C in ice-cold PBS2+ and 0.1 M glycine. Cells were solubilized in RIPA buffer (10 mM Tris, pH7.4, 150 mM NaCl, 1.0 mM EDTA, 0.1% (W/V)
SDS, 1.0% (V/V) Triton X-100 and 1.0% (V/V) Sodium deoxycholate) containing protease inhibitors (1.0 mM PMSF and 1.0 μg/ml protease cocktail). Biotinylated and non-biotinylated proteins were separated from equal amounts of cellular protein by incubation with 50 μl of 50% slurry of immobilized streptavidin-conjugated beads (Pierce, Rockford, IL) overnight with constant mixing at 4°C. Unbound proteins (supernatant) were saved for later co-immunoprecipitation experiment. Proteins bound to streptavidin beads were eluted in biotin elution buffer. Biotinylated and non-biotinylated samples were applied to protein A/G PLUS-agarose (Santa Cruz Biotechnology, Santa Cruz, CA) for co-immunoprecipitation. For nuclear biotinylated proteins, cells were firstly incubated with 1.0 mg/ml sulfo-NHS-SS-biotin (Pierce, Rockford, IL) before treatment. Cells were then treated with 50 mM glutathione to cleave all cell surface biotin, and nuclei were extracted from cell lysates [110]. After incubation with immunopure-immobilized streptavidin-conjugated beads (Pierce, Rockford, IL), beads were washed four times with RIPA buffer. The bead pellets were boiled in sample buffer and subjected to Western blot analysis.

### 4.4.3 Purification of Nuclei

Cells were gently rinsed twice with ice-cold PBS, and scraped in 1 ml of solution 1 (10 mM Hepes, pH 8.0, 10 mM KCl, 1.5 mM MgCl$_2$, 1 mM DTT, proteinase inhibitor and PMSF) per 10-cm plate. Cells were left on ice for 15 minutes to swell. Then 1% NP-40 was added and cells were centrifuged at 8,000 x g briefly. Pellets were resuspended in 175 μl of solution 2 (20 mM Hepes, pH 8.0, 1.5 mM MgCl$_2$, 25% glycerol (V/V), 420 mM NaCl, 0.2 mM EDTA, 1 mM DTT, proteinase inhibitor and PMSF) and were homogenized by four passages through a 25-gauge needle. The nuclear suspension was agitated at 4°C for 30 minutes and then was centrifuged at 16,000 x g for 5 minutes at 4°C. The supernatant is the nuclear extract.
4.4.4 Immunofluorescence

Purified GAPDH (Sigma, St. Louis, MO) was labeled with HiLyteFluo555 labeling kit (AnaSpec Inc., Fremont, CA) following manufacture’s instruction. HEK-293T cells expressing HA-GluR2 and GluR1 constructs were sub-cultured into chamber slides (Nalge Nunc, Rochester, NY) 24 hours after transfection. After another 24 hours, cells were used for experiments.

For GAPDH internalization, cells were treated with 100 μM glutamate and 100 nM HiLyteFluo555-labelled GAPDH for 30 minutes in culture medium at 37°C. Cells then were washed with cold PBS and fixed with 4% paraformaldehyde. After blocking with 2% BSA, 0.2% Triton-100 in PBS for 2 hours at room temperature, cells were incubated overnight with a rat-anti-HA antibody (Roche diagnostics, Indianapolis, IN) diluted in 1%BSA, 0.2% Triton X-100 PBS (1:500). After washes, cells were incubated for 2 hours with 1:100 diluted Cy2-conjugated anti-rat IgG secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) and 1 μM Topro3 (Invitrogen, Carlsbad, CA) (for nuclear staining). Following washes, slides were mounted with anti-fading fluorescent mounting medium (Invitrogen).

For HA-GluR2 internalization, surface HA-GluR2 was labeled in living cells by 30 minutes incubation at 37°C with a rat anti-HA monoclonal antibody (Roche) (10 μg/ml in culture medium). After washout of the antibody, cells were treated with 100 μM glutamate for 30 minutes in culture medium at 37°C. Cells were then washed with cold PBS, fixed with 4% paraformaldehyde, and blocked with 2% BSA in PBS without permeating reagent. HA-GluR2 remaining on the plasma membrane surface was stained with Cy2-conjugated anti-rat IgG (Jackson ImmunoResearch) diluted in 1% BSA in PBS for 2 hours. Cells were subsequently treated for 1 minute with 100% methanol, and then stained with Cy3-conjugated anti-rat IgG.
(Jackson ImmunoResearch) (for internalized HA-GluR2) and 1 μM Topro3 (Invitrogen) (for nuclear staining) diluted in 1% BSA, 0.2% Triton X-100 PBS for another 2 hours.

For the co-internalization of surface HA-GluR2 and GAPDH, surface HA-GluR2 was labeled in living cells as mentioned above. After washout of the antibody, cells were treated with 100 μM glutamate and 100 nM HiLyteFluo555 labeled GAPDH for 30 minutes in culture medium at 37°C. After wash and fixation, cells were blocked under permeating condition with 2% BSA, 0.2% Triton-100 in PBS and stained with Cy2-conjugated anti-rat IgG (Jackson ImmunoResearch) (for both HA-GluR2 remaining on the cells surface and internalized HA-GluR2) and 1 μM Topro3 (Invitrogen) (for nuclear staining). Subcellular localization of fluorescently labeled HA-GluR2 and GAPDH were examined with a LSM510 confocal microscope (Zeiss, Germany).

As for the immunohistochemistry for rat brain section, rat brain cryostat sections (20 μm) were collected on Fisher Tissue Path Superfrost plus Gold Slides (Fisher Scientific Company, Ottawa, Canada). Tissue sections were blocked for 2 hours at room temperature with blocking buffer (PBS with 0.2% Triton X-100 and 2% BSA), and incubated overnight with 1:200 diluted anti-GAPDH mouse monoclonal antibody (Abcam, Cambridge, MA) and 2 hours with 1:100 diluted Cy3-labeled anti-mouse IgG secondary antibody (Jackson ImmunoResearch Laboratories). Following washes, sections were then stained with 1:100 diluted Fluo488-conjugated anti-NeuN antibody (Millipore, for neuronal staining) and 1 μM ToPro3 (Invitrogen, for nuclear staining) for another 2 hours. Sections were washed and mounted with anti-fading fluorescent mounting medium (Invitrogen).
4.4.5 Ischemia Induction in Rats

The four vessel occlusion ischemia model was performed on male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA), weighing between 300 and 400g, using a modified version of the classical method of Pulsinelli and Brierley [111]. Both vertebral arteries of the test subjects were permanently occluded by electrocauterization (Matin MD 62 electrosurgical unit, Harvard Apparatus) and the common carotid arteries were loosely snared with silk threads. On the following day, animals were injected with 5 nmol TAT-GluR2NT1-3-2 or TAT-scrambled peptide either 1 hour before or 2 hours after the induction of ischemia. Common carotid arteries were occluded with aneurysm clips for 10 minutes. After 2 minutes blood flow occlusion, righting reflex and pupil dilation appeared and laser Doppler perfusion monitor was applied to directly and non-invasively measure the brain blood flow to confirm ischemia. Sham control animals received same surgical preparation and recovery paradigms, but no transient carotid occlusion. After a 5 day recirculation period, animals were sacrificed and rat brains were fixed with 4% paraformaldehyde in PBS buffer and cryoprotected by infiltration with 20% sucrose solution. 20-µm hippocampal coronal sections were cut and applied for cresyl violet (Sigma, St. Louis, MO) staining. The number of cresyl violet-positive neurons within the reticule field was counted in 6 sections for each animal under a light microscope.

4.4.6 MCAO methods

Transient focal cerebral ischemia (90 minutes) is induced by right intraluminal middle cerebral artery occlusion (MCAO) as described previously [71, 112]. Briefly, rats were anesthetized and MCAO was achieved by introducing a 3–0 monofilament suture into the middle cerebral artery via the internal carotid artery. Body temperature was maintained at 37.0 ± 0.5°C using a heating lamp and a rectal thermometer during ischemia and reperfusion. 24 hours after
MCAO onset, rats were deeply anesthetized and sacrificed. The brains were cut into 1-mm-thick coronal sections and stained with 0.05% 2, 3, 5-triphenyltetrazolium chloride for 30 minutes at 37°C, followed by overnight immersion in 10% formalin. The infarct zone was demarcated and analyzed Image J (Image J, Bethesda, MD) software. 2 hours after MCAO and 10 minutes before the animals were killed, two neurological tests were performed: The postural reflex test to examine upper body posture [72] and the forelimb placing test to examine sensorimotor integration in forelimb placing responses to visual, tactile, and proprioceptive stimuli [73] were performed to grade neurological function on a scale of 0–12 (0, normal; 12, worst). In the treatment groups, a single injection of 4 μl 10mM TAT-GluR2NT or TAT Scrambled peptide was infused intracerebroventricularly (I.C.V) (AP: –1.0 mm; LM: –1.4 mm; DV: –3.6 mm from Dura) 2 hours before, 30 minutes after the onset of MCAO, whereas sham operation group underwent all the surgical procedures without insertion of suture.
References


Chapter 5

5. Conclusions and future directions

5.1 Conclusions

It has been three decades since the concept of receptor-receptor interaction introduced in the early 1980s. During this period, numerous studies have been focusing on investigating the biochemical, functional as well as the pathological implications of the receptor-receptor interaction. More importantly, in both animal and human studies, there has been considerable evidence demonstrating the important roles of receptor-receptor interactions in neuropathological diseases, which involve dysfunctions in multiple neurotransmitter systems. The current clinical treatments of neuropathological disease mainly depend on the direct blockade of specific receptors to remit the symptoms, which blocks both physiological and pathological pathways and may therefore exert severe side effects. Thus, instead of simply blocking one dysfunctional receptor system, the regulation of the receptor-receptor interaction involved in the neuropathological disorders has become a potential target for the new drug development (Chapter 1).

In the present study, we identified a potential role for D2R dimerization in the pathology of schizophrenia (Chapter 2). This conclusion is based on our observations of enhanced D2R dimers in the brain tissue of schizophrenia patients, in the animal model of AISS and in cell cultures. Moreover, we have demonstrated that the physical coupling between D2R and DAT is important for this amphetamine-induced up-regulation of D2R dimerization. Although the mechanisms regulating this process remain unknown, a treatment focusing at reversing the enhanced D2R dimerization and/or interrupting D2-DAT interaction may become a novel therapeutic target for the development of antipsychotics.
In the next chapter of this study, we provide the first direct evidence implicating the D1-D2 receptor complex in the pathology of depression, and also identify an interfering peptide that can disrupt the D1-D2 interaction and exert antidepressant-like effects (Chapter 3). This conclusion is based on the observation that the enhanced coupling between D1 and D2 receptors in the brain tissue of depression patients, and the administration of an interfering peptide that disrupts the D1-D2 receptor complex exerts antidepressant-like effects in both short- and long-term depression animal models. It should be noted that only one third of patients who receive the single trial of the current antidepressant treatment achieve full remission of their symptom. Even with multiple antidepressant trials, there are still at least 10-15% of patients who continue to experience persistent depressive symptoms and few alternatives have been available for the treatment of resistant symptoms. Therefore, the interfering peptide in our study that exerts antidepressant-like effects may provide a novel therapeutic strategy for the treatment of depression.

In Chapter 4 of this study, we identify a previous unrecognized direct protein-protein interaction between GluR2 and GAPDH and provide evidence demonstrating the important role of this GluR2-GAPDH interaction in the pathology of ischemic stroke. This conclusion is based on the observation that the activation of AMPAR promotes GluR2-GAPDH complex formation and leads to the nuclear translocation of the complex, where GAPDH binds to p53 and initializes the p53-dependent cell death pathway. More importantly, the administration of the interfering peptide interrupting the GluR2-GAPDH interaction mitigates the cell death in both global- and focal-ischemic animal models. In addition to stroke, considerable evidence have also demonstrated that patients with chronic neuropsychiatric diseases, such as schizophrenia and depression mentioned above, may be at particular risk of neuronal loss through cell death, which involves glutamate excitotoxicity and altered calcium signaling pathways. Recently, the
development and clinical testing of glutamate receptor antagonists have received intense attentions in the treatment of this field. It is worth noting that these glutamate receptor antagonists do not only inhibit ischemia-induced excitotoxicity, but also block the excitatory neurotransmission that is important for maintaining normal brain function. Therefore, the discovery of the GluR2-GAPDH interaction may provide a new avenue for the development of a complementary therapeutics for the cell loss in neuropathological diseases.

5.2 Future Directions

5.2.1 D2R dimerization in schizophrenia

Previous studies indicate that D2R are expressed as monomers and dimers in cell lines and in mammalian brain tissue [1-4]. Furthermore, D2R have also been reported as dimers in variety of neurological diseases such as Alzheimer’s Parkinson’s and Huntington’s disease [5, 6]. In Chapter 2 of the present study, we observed enhanced expression of D2R dimers in post-mortem striatal tissue of schizophrenia patients, which helps shed more lights on understanding the pathophysiological role of D2R dimerization. However, it should be noted that the AMPH-induced D2R dimerization may be an epiphenomenon rather than a causative factor. The underling molecular mechanism is still not clear and more questions are waiting to be answered.

It is now generally accepted that GPCRs can exist as dimers or as part of larger oligomeric complexes. Great efforts have been put into studying the composition, topology and pharmacological properties of GPCR oligomers. Previous studies have shown that D2 receptors form dimers through multiple sites including transmembrane domain 4, whose physiological or pathological effects are still unknown [7]. Thus, further studies should be carried out to identify the exact interacting sites that are responsible for the AMPH-induced D2R dimerization to facilitating the development of specific interfering peptides.
Previously, our lab reported that D2R directly interacts with DAT, and the D2R-DAT protein complex formation enables D2R to up-regulate DAT-mediated dopamine uptake by recruiting DAT to the plasma membrane [8]. In the present study, we have shown that an interfering peptide that disrupts the D2R-DAT coupling can block the AMPH-induced up-regulation of D2R dimerization, suggesting the important role of D2R-DAT protein-protein interaction in this phenomenon. Based on the observation in the present study and the fact that AMPH increases dopamine concentration in the synaptic cleft by reversing DAT-mediated dopamine uptake, we would speculate that the AMPH-induced excess dopamine in the synaptic cleft may contribute to the formation of D2R dimers and to the alteration of D2R pharmacological properties. Further experiment focusing on studying the molecular mechanisms involved is needed to clarify the role of D2R-DAT interactions in the AMPH-induced D2R dimerization.

As discussed in Chapter 1, aside from DAT, D2R also interacts with many other neurotransmitter receptors or channels, such as D1R [9-11], D3R [12] and A2A receptors [13]. Several dopamine receptor interacting proteins have also been identified to be directly and indirectly associated with D2R, including protein 4.1N/B/G, FilaminA, Spinophilin GIPC, CAPS1, ZIP, NCS-1 and GRK2 [14-20]. It is worth noting that the AMPH–induced increase in D2R dimers seems to have bands that are more intense than the monomer bands. If the DAT is the only molecule required for this process, it may appear surprising that the DAT in such a small fraction of TH-positive neurons accounts for a large increase in DRD2 dimers in the cultured striatal neurons. Thus, there is a high probability that other additional molecules may be involved in D2R dimerization, and future investigations are needed to discover other potential players in the AMPH-induced D2R dimerization.
Although we have demonstrated that the ability of the interfering peptide TAT-DAT_{NT1-1} to block the AMPH-induced D2R dimerization, whether disrupting the D2-DAT interaction can exert antipsychotic effects \textit{in vivo} is still unknown. Due to the similarity between schizophrenia and amphetamine/apomorphine psychosis, the AISS and PPI have been widely used as animal models of schizophrenia. Although these dopamine-linked behaviors are not specific for or uniquely prominent in schizophrenia, they can be at least detected and precisely quantified in non-human species and have been useful in screening drugs with a predicted mechanism of action [21, 22]. It should be noted that the majority of AMPH abusers do not go on to develop a chronic schizophrenic-like syndrome, and only about one-third of schizophrenia patients do have AMPH abuse history. Thus, future experiment is needed to examine the antipsychotic effects of the interfering peptide TAT-DAT_{NT1-1} in AISS or other animal models such as DISC1 mutant mice, which may help us to better understand the underlying mechanisms and open a new door for the treatment of schizophrenia.

5.2.2 D1-D2 in depression

5.2.2.1 Functional roles of D1-D2 interaction in depression

Dopamine, a neurotransmitter that plays a crucial role in multiple functions in CNS [23] by exerting its physiological effects via five dopamine receptor subtypes, termed D1 to D5. D1 receptors preferentially couple to Gs or Golf proteins stimulating the activity of adenylate cyclase and PKA-dependent pathway, while D2 receptors couple to Gi/o protein family [23]. Although dopamine D1 and D2 receptors belong to distinct subfamilies of dopamine receptors, several lines of evidence indicate that they are functionally linked. For example, behavioral studies have shown that co-stimulation of the D1 receptor is important for D2 agonists to produce maximal locomotor stimulation [24] and that activation of both D1 and D2 receptors is
required to augment the acute effects of cocaine action [25]. Biochemical and electrophysiological evidence has also supported D1-D2 receptor synergism. Combined administration of specific D1 and D2 agonists potentiates immediate early gene expression [26, 27]. Also, long term depression of synaptic transmission after dopamine depletion could be restored by dopamine or co-administration of specific D1 and D2 agonists but not by either selective agonist alone [28]. However, the mechanism for the D1-D2 receptor interaction in these studies showing functional linkage has not been elucidated until the recent discovery of the D1-D2 receptor complex by George et al [10]. The D1-D2 receptor complex induces calcium release via a Gq-dependent pathway distinct from the classical G proteins coupled to D1 and D2 receptors, and the calcium mobilization further activate CaMKIIα, which leads to an enhanced BDNF expression. Recent studies have also shown that the D1-D2 receptor complex-mediated calcium signal can be desensitized by agonist occupancy of either receptor, which may be caused by the co-internalization of the receptor complex [29, 30]. Moreover, the sensitivity and functional activity of D1-D2 complex have also been found to be upregulated in some neuropsychiatric diseases [31].

In the present study, we have observed that the co-activation of the D1-D2 receptor complex induces intracellular calcium release, which can be blocked by disrupting the D1-D2 interaction. However, this experiment was only done in transfected HEK293T cells co-expressing D1 and D2 receptors, future experiment is needed to examine whether blocking the D1-D2 interaction by the interfering peptide can inhibit the intracellular calcium release and downstream CaMKIIα phosphorylation in a relevant cellular milieu, such as cultured neurons and brain tissue. Furthermore, whether the interfering peptide exerts its antidepressant-like effects via regulating the trafficking, desensitization and/or sensitivity of the D1-D2 receptor
complex is still unknown. Therefore, future studies are required to unveil the underlying molecular mechanisms to better understand the role of the D1-D2 interaction in depression.

5.2.2.2 Application of the interfering peptide in other depression animal models

In the present study (Chapter 3), we have examined the antidepressant-like effects of the D1-D2 interfering peptide in LH model. In this model, inescapable and uncontrollable stress is used to induce depressive-like learned helplessness behavior, while escapable and controllable stress has no serious consequences [32]. The central role of controllability in the learned helplessness paradigm was observed early on and appears to be a factor in the conditions leading to human depression [33]. As in humans, the rat medial prefrontal cortex is involved in detecting controllability and the pathogenesis of learned helplessness which supports the usefulness of this paradigm as an animal model for depression [34]. Other than the LH model, several well established depression animal models are also available including chronic mild stress (CMS), and psychosocial stress. Similar to the LH model, most of them use stress to induce depressive-like states. In the CMS model chronic unpredictable mild stress like soiled cages, changing cage mates or unpredictable feeding times is used to induce depressive-like behavior. In this model stressors are applied over a considerable time from 3 weeks to 3 months, thus mimicking long-lasting stress leading to human depression [35]. The model of chronic psychosocial stress of the tree shrew combines uncontrollability and chronicity of the stressor in a paradigm of natural relevance. Male tree shrews defend their territories against intruders. When two adult males are housed together in one cage, they will fight and establish a social hierarchy with a dominant and a subordinate male. The subordinate experiences chronic stress as long as it lives in visual and olfactory contact with the dominant male [36]. This chronic defeat stress model overcomes the limitation of the acute depression model in that the social avoidance induced in the test can be
treated with chronic, but not acute, antidepressant administration. Another advantage of the social defeat model is that it involves a social form of chronic stress, which may be relevant to stress-induced psychopathology in humans [62]. Due to the fact that animals couldn’t express feeling by language, sucrose consumption model can be used to examine the pleasure level of animals. Since different depression animal models possess distinct characteristics, future experiment is needed in other depression models to study the antidepressant-like effects of the D1-D2 interfering peptide more comprehensively.

5.2.3 GAPDH-GluR2 in ischemia

5.2.3.1 Intracellular events that follow the GAPDH-GluR2 complex internalization.

In Chapter 4 of the present study, we have shown that GAPDH is internalized by binding to the extracellular N terminus of GluR2, and the protein complex may therefore be in a vesicle. Thus, it would be logical to ask how the GAPDH-GluR2 complex gets out of the vesicle and into the cytoplasm for entering the nucleus.

There are many possibilities for this question. First, the complex may be transported to the nucleus via a retrograde vesicle transport mechanism leading to the fusion of the vesicle with ER or nuclear membranes or via mechanisms recently proposed for the nuclear translocation of another plasma membrane receptor, the EGF receptor [37, 38]. Second, the GAPDH-GluR2 complex formation in the vesicle may lead to the activation of lysosome in the vesicle that breaks the vesicle and release the GAPDH-GluR2 into the cytoplasm. The fact that GAPDH does not contain a NLS sequence [39] indicates the possibility that either GluR2 or another molecule involved in the GAPDH-GluR2 complex contains a NLS sequence. Recent studies have suggested that GAPDH binds to Siah1, which contains nuclear localization signal (NLS), to
promote GAPDH translocation into the nucleus [39]. The association with GAPDH stabilizes Siah1 and thereby enhances Siah1-mediated proteolytic cleavage of its nuclear substrates, such as N-CoR, and triggers apoptosis. It is clear that much more needed to be done for a better understanding of the detailed molecular pathways through which the GAPDH-GluR2 complex translocates from the plasma membrane to the nucleus.

5.2.3.2 p53-dependent cell death pathway.

P53, a key modulator of cellular stress responses was previously implicated in the glutamate-mediated excitotoxicity [40-42]. Considerable evidences also demonstrate that p53 is involved in neuronal death in both in vivo cerebral ischemic animal models [43-48] and in vitro hypoxic conditions [49]. Furthermore, a previous study showed an interaction between GAPDH and p53 involved in neuronal apoptosis [50]. More importantly, recent studies have demonstrated that the p53 deficiency or applications of p53 inhibitors can markedly attenuate brain damage in various stroke models [51]. However, since p53 plays a crucial role in tumor suppression by sensing genotoxic stress and preventing proliferation of damaged cells through inducing cell cycle arrest, senescence and apoptosis [52], clinical application of p53 inhibitors for stroke treatment may also induce an increased risk of cancer.

In the present study, we have demonstrated the enhanced GAPDH-p53 interaction in ischemia animal model, and the disruption of the GAPDH-p53 interaction considerably attenuates the AMPAR-mediated cell death. The observation that the pretreatment of the p53 inhibitor PFTα substantially inhibits glutamate-induced cell death in HEK293T cells expressing GluR1/2 subunits further confirms the important role of p53 in this process. However, the molecular mechanism is still unknown. Considerable evidences have shown that p53 induces neuronal apoptosis via the transcriptional pathway by up-regulating the expression of its target
gene p21^{WAF} [53, 54], Peg3/Pw1 [55] or p53-up-regulated modulator of apoptosis (PUMA) [56, 57]. Furthermore p53 has also been reported to be able to disrupt NF-κB binding to p300 and block NF-κB-mediated survival signaling [58]. In addition, the transcription-independent pathway mechanism is also of great importance. In this pathway, p53 translocates to mitochondrial and mediates the release of Cytochrome c [45, 59]. Therefore, further investigation of the p53-mediated cell death signaling pathway involved in the AMPAR-mediated cell death will make great contributions for the development of novel therapeutic tools for the ischemic stroke.

5.2.3.3 Therapeutic windows and the significance in clinical trials.

Given the fact that very few effective pharmacological options are effective, it is of urgent importance to develop novel therapeutic strategies for the treatment of ischemic stroke. In the present study, we have examined the neuronal protective effects of the TAT-GluR2_{NT1-3-2} peptide in both global and focal ischemia animal models with satisfactory results. However, it still would be a long road as the peptide treatment moves from bench top to bedside. As shown in Figure 4-17, we have only assessed the cell death at 5 days after global ischemia. A longer time point is needed to rule out the possibility that the treatment with the peptide delays rather than prevents cell death.

One of the well-known treatment tools of ischemic stroke is thrombolytics, such as alteplase and tenecteplase. Thrombolytics have been a significant advance in the treatment of ischemic stroke. However, the efficacy of thrombolytics necessitates early intervention (within 3 hours of ischemic episode), which has significantly limited their practical use in much of the world [60, 61]. As a result, there continues to be a significant unmet need for acute pharmacological treatments that go beyond the use of thrombolytics. The similar issue also
occurs in our study. The TAT-GluR2\textsubscript{NT1-3-2} was administrated only 2 hours after global ischemia is induced to observe the neuronal protective effects (Figure 4-17). In order to further assess the clinical relevance of this observation, post-treatment should be extended to determine the temporal window in which the peptide is effective.

In the focal ischemia animal model, we assessed the lesion volume at 24 hours after ischemia in order to observe the protective effect of the interfering peptide. It should be noted that the damage may not be completed at that time point, and longer survival times are needed to rule out that the expression of the damage was delayed rather than prevented. Similar as in the global model, in order to assess the clinical relevance of the observations in focal ischemia model, post-treatment time must be extended to determine the temporal window in which the peptide is effective.

5.3 Final Thoughts

One last consideration in this thesis is that of the significance of those interfering peptides in attenuating clinical symptoms presented in neuropathological diseases. It is frequently difficult to contribute more than a minor “piece of the puzzle” to the vast array of knowledge surrounding mechanisms involved in the pathophysiology of neuropathological disorders. Thus, any one observation can seem diminished as it will likely only address a minor contributor to the whole story. Whether the effects that we have observed in the present study are a dominant contributor is still unknown, particularly in the clinical context. However, given the fact that the current available clinical drugs often come with inevitable side effects and limited therapeutic windows, our study focusing on regulating the receptor-receptor interactions may provide a novel therapeutic tool for the treatment of neuropathological disorders.
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