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The Role of NR2A – and NR2B- Containing NMD^A Receptors in Innate Anxiety

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THE ROLE OF NR2A- AND NR2B- CONTAINING NMDA RECEPTORS IN INNATE ANXIETY

This thesis is submitted to the
Faculty of Graduate and Postdoctoral Studies
as a partial fulfillment of
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By

Xuesheng Li

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ABSTRACT

Anxiety disorders are very common mental health diseases affecting millions of people worldwide and cost billions of dollars in medical care each year. However, the exact biological mechanism underlying the occurrence of anxiety disorders is still not clear. Accumulating studies have shown that *N*-methyl-D-aspartate (NMDA) type of glutamate receptors (NMDARs) are involved in anxiety disorders in animal models, but the roles of NR2A- and NR2B-containing NMDARs in anxiety disorders remain controversial, and it is completely unknown whether NMDAR-mediated LTP or LTD are involved in anxiety disorders. This project was designed to further investigate the roles of NR2A- and NR2B-containing NMDARs and NMDAR-mediated LTP and LTD in anxiety disorders. The effects of NR2A specific antagonist NVP-AAM077 (1.2mg/kg and 2.4mg/kg), NR2B specific antagonists Ro25-6981 (6mg/kg) and ifenprodil (5mg/kg), and LTD blocking peptides Tat-GluR2-3Y and Tat-NR2B9c (1.5 μ mol/kg) were assessed with Elevated Plus-Maze (EPM) and/or modified Novelty Suppressed Feeding (NSF) tests in rats. The effect of bilateral infusion of Ro25-6981 (100 μ M, 1 μ l) into the basolateral amygdaloid nuclei (BLA) was also examined. Our results showed that NVP-AAM077 and LTD-blocking peptides produced no significant effects on innate anxiety both in the EPM and NSF tests. Systemic administration of Ro25-6981 and ifenprodil produced anxiogenic effect, whereas intra-BLA injection of Ro25-6981 produced anxiolytic effect in the EPM test. These results reveal that NR2A-containing NMDARs and NMDAR-mediated LTP are not involved in anxiety disorders, and that NR2B-containing NMDARs play an important role in the development of anxiety disorders through an unknown pathway other than LTD production.

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LIST OF ABBREVIATIONS

AC	Adenylyl cyclase
AMPARs	α -amino-3-hydroxy-5-methyl-4-isoxazole propionate-preferring receptors
BLA	Basolateral amygdaloid nuclei
CaMKII	Calcium-calmodulin regulated kinase II
CNS	Central nervous system
CREB	cAMP response element binding
EPM	Elevated Plus-Maze
GAD	Generalized anxiety disorder
HFS	High-frequency stimulation
LFS	Low-frequency stimulation
LTD	Long term depression
LTP	Long term potentiation
MAPK	Mitogen-activated protein kinase
mGluR	Metabotropic Glutamate receptor
NMDARs	<i>N</i> -methyl-D-aspartate-preferring receptors
NSF	Novelty-Suppressed Feeding test
NVP- AAM077	[(R)-[(S)-1-(4-bromo-phenyl)-ethylamino]-2,3-dioxo-1,2,3,4-tetrahydro-quinoxalin-5-yl)-methyl]-phosphonic acid
OCD	Obsessive-compulsive disorder
PBS	Phosphate-buffered saline
PD	Panic disorder
PKA	Protein kinase A

PKC	Protein kinase C
PSD	Post synaptic density
PTSD	Post-traumatic stress disorder
Ro25-6981	(R-(R*,S*)-a-(4-hydroxyphenyl)-b-methyl-4-(phenylmethyl)-1-piperidinepropanol

1. INTRODUCTION

1.1 Anxiety

1.1.1 General Anxiety and Anxiety Disorders

Anxiety is a painful uneasiness of mind, usually over an anticipated ill, an abnormal apprehension and fear often accompanied by physiological signs such as sweating and increased pulse, by doubt about the nature and reality of the threat itself and by self-doubt (American Psychiatric Association, 2000).

Everyone experiences feelings of anxiety at some point in their lives. Whether it is the birth of a first child, the sudden loss of a job, in preparation for a major medical procedure or for the first public speech, everyone experiences occasions where their stress level can become overwhelming. As a close relative of excitement, anxiety can be best described in terms of worry, a general troubled or uneasy feeling of apprehension. This so-called general or ordinary anxiety is relatively mild, brief or periodic, caused by a realistic stressful event (such as speaking in public or a first date). It is a normal response to stress and helps one deal with a tense situation.

Anxiety disorders, however, are altogether different from the above mentioned ordinary anxiety. The worry and fear experienced by people suffering from an anxiety disorder is persistent, lasting at least for six months, and habitual, often initiated by unrealistic situations or thoughts (American Psychiatric Association, 1994). In addition, this worry is seemingly uncontrollable and often interferes with their ability to concentrate or conduct normal working and family activities day to day (American Psychiatric Association, 1994).

The symptoms of anxiety disorders vary by type, but the key feature of anxiety

disorders is increased fearfulness accompanied by subjective as well as objective manifestations. The subjective manifestations range from a heightened sense of excessive, irrational fear and dread to a deep fear of impending disaster and death. The objective manifestations include racing heart, increased blood pressure, avoidance behaviour and signs of restlessness, heightened responsiveness, palpitations, tremor, sweating, dry mouth, nausea, muscle tension and a desire to run or escape (American Psychiatric Association, 2000).

1.1.2 Subtypes of anxiety disorders

Based on clinical symptom clusters, time course and therapeutic responses, anxiety disorders can be subdivided into several types. According to the most widely used classification of psychiatric disorders in Diagnostic and Statistical Manual of Mental Disorders 4th edition (DSM-IV), primary anxiety disorders are classified as follows (American Psychiatric Association, 1994).

1. Generalized anxiety disorder (GAD), is characterized by a state of excessive anxiety and worry about a number of events or activities lasting for more than six months. Neurovegetative symptoms are often present, but are relatively minor.

2. Panic disorder (PD), is characterized by recurrent panic attacks, either unexpected or associated with particular situations. There are three groups of symptoms: reexperiencing, avoidance and numbing, and arousal. Panic attacks are sudden surges of intense fear or terror, desire of fleeing and feeling of imminent death, going crazy or losing control. These subjective symptoms are accompanied by some of the following physical symptoms, such as palpitations, increased heart rate, hypertension, shortness in

breathing or feeling of choking or chest pain, trembling or shaking, sweating, urge to void the bladder and increased peristalsis. This leads to worry about the next attack or anticipatory anxiety, and avoidance of places or situations where a panic attack would be embarrassing. Ultimately, generalized avoidance or agoraphobia may ensue.

3. Obsessive-compulsive disorder (OCD), is characterized by persistent intrusive, distressing thoughts ideas, impulses or images (obsessions), and/or stereotyped or ritualized behaviour such as hand washing, ordering or checking (compulsions) that must be performed repetitively in order to alleviate intense anxiety.

4. Specific phobias are marked and persistent irrational fears of clearly discernible objects (animals, blood, pointed instruments) or situations (heights, flying, closed environments).

5. Social phobia (also called social anxiety disorder) is the marked anxiety experienced in social or performance situations, such as speaking in public, going to parties or being in a classroom. The provoked anxiety responses by the social exposure include tremors, sweating, palpitations, gastrointestinal discomfort, diarrhea, muscle tension, blushing or confusion.

6. Post-traumatic stress disorder (PTSD) develops after a terrifying experience that involved physical harm or the threat of physical harm (such as war, natural disaster, child abuse or rape). People with PTSD are suffering the flashbacks of the terrifying experience, persistent frightening thoughts and memories, and they may startle easily, become emotionally numb (especially in relation to people with whom they used to be close), lose interest in things they used to enjoy, have trouble feeling affectionate, be irritable, become more aggressive, or even become violent.

Anxiety disorders are the most common psychiatric problems, found in about 40 million American adults, i.e. nearly 20% of the general population (Kessler et al., 2005a,b). As a whole, anxiety disorders cost the United States between 42-46 billion dollars a year in direct and indirect healthcare, which is a third of the yearly total mental health bill of 148 billion dollars (Kessler et al., 2005a,b). In Canada, anxiety disorders affect approximately 12% of the population during a one-year period, and the direct health care cost and other associated costs are also considerable (Offord et al., 1996). Anxiety disorders affect men and women of every age group, but women experience anxiety and stress-related problems at a higher rate than men (American Psychiatric Association, 2000). Commonly, anxiety disorders occur along with other mental or physical illnesses, such as depression, alcoholism and other types of substance abuse, which may mask the symptoms of anxiety disorders or make them worse (American Psychiatric Association, 2000).

To date, the exact cause of anxiety disorders is unknown, although they are generally believed to be triggered by a combination of some risk factors including personality characteristics, genetics/biology, developmental and environmental factors (Millon et al., 1999).

1.1.3 Animal models of anxiety

Over the past few decades, it has been intensively studied of the neurobiological aspects of anxiety disorders (Nemeroff, 2004) in order to get best understanding the mechanism of anxiety disorders and obtain effective treatment strategies for the patients (Paterson et al., 2001). Because of the ethical and some similar reasons it is impossible

to study the impact of some stress factors in humans, or to test the effects of newly developed anti-anxiety medications. Thus some animal models of anxiety were developed.

Experimentally anxiety models can be subdivided into two categories. One category includes open field test (Moyaho et al., 1995), elevated plus-maze test (Andrade et al., 2003), light-dark box test and so on (Flint, 2003). These models are used to examine the animals' innate anxiety, which is induced by acute aversive pharmacologic, novel or stress factors and no learning and memory formation process involved. The other category is the fear conditioning (cued or contextual) model (Flint, 2003), in which after pairing a conditioned neutral stimulus with an unconditioned aversive stimulus repetitively delivering the neutral stimulus alone will elicit anxiety-like behaviours. It is a very reliable task to study the learned anxiety that involves the learning and memory formation process.

1.2 NMDA receptors

Chemical synaptic transmission is a widely used means of fast communication between neurons. In the mammalian central nervous system (CNS), the simple amino acid L-glutamate is the major excitatory synaptic transmitter (Dale and Kandel, 1993). Its release from presynaptic nerve terminals produces a brief chemical signal that is converted back into an electrical event when glutamate molecules bind to ionotropic glutamate receptors. These receptors, mainly located in the postsynaptic membrane, contain an integral cation-selective ion channel that allows a net inward current, resulting in postsynaptic depolarization. Ionotropic glutamate receptors fall into three

broad classes: the α -amino-3-hydroxy-5-methyl-4-isoxazole propionate-preferring receptors (AMPA receptors), the kainate-preferring receptors and the *N*-methyl-D-aspartate-preferring receptors (NMDARs) (Palmada and Centelles, 1998). At many synapses AMPARs act as the main initial charge carriers during excitatory transmission, while NMDARs generate a slower current with a substantial Ca^{2+} component, which can modulate the activity of second messengers and intracellular enzymes (Sattler et al., 1999). The enormous interest in the NMDARs derives, at least in part, from the fact that investigation of their elementary properties has continued to reveal fundamental information about their novel role in central synaptic transmission, plasticity and certain pathological changes (Cull-Candy and Brickley, 2001).

1.2.1 Diversity of NMDAR subunits and isoforms

Studies, including electrophysiological, ligand binding, molecular biological and morphological experiments, have revealed a large diversity of NMDAR subunits and isoforms within the CNS (Hollman, 1999). To date, three main families of NMDAR subunits have been identified: NR1, NR2 and NR3.

The NR1 family of subunits is encoded by a single gene (Nakanishi et al., 1998). However, post-translational modification occurs at three independent splice sites on the gene and generates eight functional isoforms. NR1 is capable of forming functional homomeric NMDARs only in *Xenopus* oocytes (Nakanishi et al., 1998). In mammalian cells, NR1 subunits need to be co-expressed with NR2 subunits to form functional receptors. Indeed, all native NMDARs are thought to contain both subunits.

The type of NR1 isoform involved in receptor formation is of importance in determining various properties of the NMDARs.

The NR2 subunit family is composed of four members: NR2A, 2B, 2C and 2D arising from separate genes (Hollman, 1999). Splice variants were reported in all NR2 subunits except NR2A. NR2 subunits were assembled exclusively as heteromers with other NR family members, most commonly with NR1 (Monyer et al., 1994). The biological and pharmacological properties of NMDARs are strongly influenced by the subtypes of NR2 involved (Sobolevsky et al., 2002).

The NR3 subunit has two isoforms, NR3A and NR3B, and only NR3A was extensively studied. NR3 subunit plays a regulatory role in functional NMDARs composed of NR1 and NR2 subunits during early development (Das et al., 1998). Coexpression of the NR3A subunit with recombinant NR1/NR2A-containing NMDARs causes a marked reduction in single-channel conductance and a decrease in the ion channel's Ca^{2+} permeability (Das et al., 1998). However, it is not yet clear how this subunit contributes to the structure or function of the native NMDARs.

1.2.2 Structure of NMDARs

Now it is widely accepted that native NMDARs are heteromeric assemblies composed of obligatory NR1 subunits together with at least one type of NR2 subunits (Laube et al., 1998; Monyer et al., 1994). Domains from the NR1 subunit form the coagonist glycine-binding site, whereas the glutamate-binding site is mapped to the NR2 subunit (Behe et al., 1999). (Figure 1.1)

Recent experiments on the stoichiometry of NMDARs support the tetrameric subunit complex model, i.e., each NMDAR is assembled with either two NR1 subunits and two NR2 subunits, or two NR1 subunits, one NR2 subunit and one NR3 subunit (Laube et al., 1998). In cells expressing only one type of NR2 subunit, NMDAR complex contains two identical NR2 subunits. There is also evidence that some native NMDARs contain two different NR2 subunits, although to date it remains unknown whether these receptors are expressed widely (Sheng et al., 1994)).

It has been confirmed that all glutamate receptors share certain structural similarities. Each glutamate receptor subunit is composed of three transmembrane domains, M1, M3 and M4, and a pore-forming domain M2, which dips into the membrane from the intracellular side and forms a re-entrant loop (Dingledine et al., 1999). There are a long N-terminal, a long C-terminal, two short intracellular loops (L1 between M1 and M2, L2 between M2 and M3) and a long extracellular loop (L3 between M3 and M4). The two globular lobes, one near the membrane part of the extracellular N-terminal and the other close to M3 domain part of L3 loop, form a ligand-binding pocket. (Figure 1.2)

1.2.3 Functional properties of NMDARs

The functional characteristics of multiprotein NMDAR complexes are determined by their subunit composition. The NMDARs appear unique among ligand-gated ion channels in that their activation requires the binding of both the excitatory neurotransmitter (glutamate) and the coagonist (glycine) (Dingledine et al., 1999). The binding site for glycine located at NR1 subunit must be occupied before the NMDARs

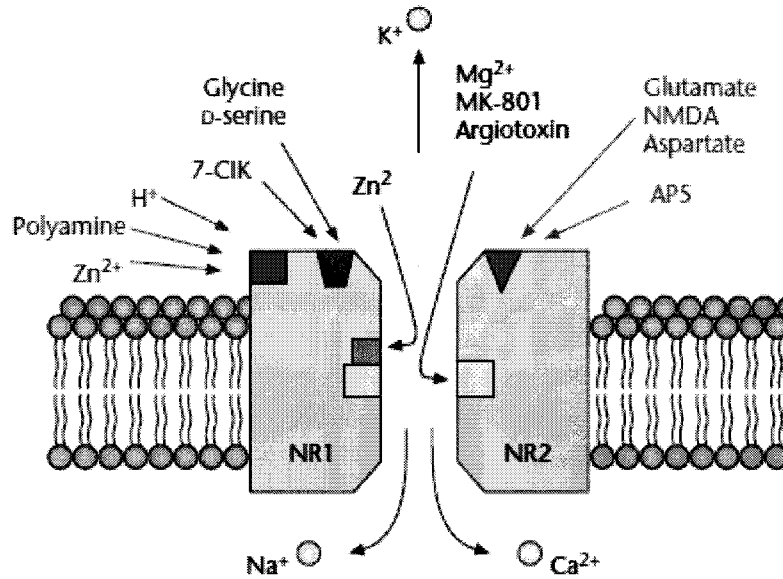


Figure 1.1 Schematic drawing of the NMDAR channel, depicting its main binding sites (Cited from Cull-Candy and Brickley, 2001).

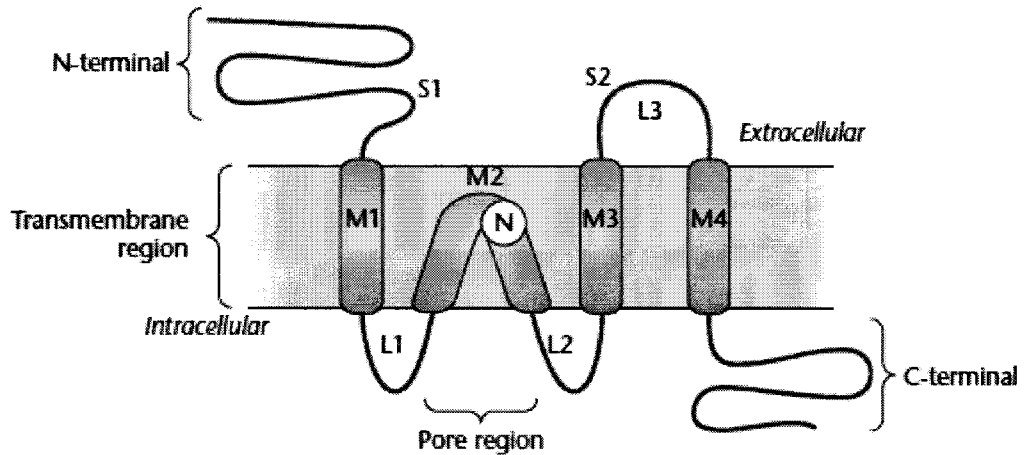


Figure 1.2 Transmembrane topology of the NMDAR subunits. (Cited from Cull-Candy and Brickley, 2001)

are activated by the agonist, glutamate or NMDA. The general idea from the analysis of kinetics of NMDARs activation is that two molecules of glutamate and two molecules of glycine must bind to native NMDARs to activate ion channel gating (Smith et al., 1992). The physiological role of modulation by the coagonist glycine is still far from clear, but this has triggered great interests of pharmacologists, who believe that the inactivation of NMDARs merely by blocking the glycine binding site will be a novel therapeutic means in reducing the neuron damage following the ischemic stroke (Liu et al., 2007).

In addition, diverse extracellular ions that affect the activity of NMDARs via binding to these receptors have been identified. In particular, at the cell's resting membrane potential, Mg^{2+} ions bind to a site located deep within the ion channel to occlude the flow of cations. This block is voltage-sensitive and is relieved or even completely removed by depolarization via AMPARs activation (Malenka and Nicoll, 1999), in accordance with channels with unusually slow activation/deactivation kinetics and a high permeability to Ca^{2+} ions (as well as Na^+/K^+). So generally NMDARs contribute little to basal synaptic transmission, but are necessary for producing different forms of synaptic plasticity. Conversely, AMPARs are the primary receptors mediating basal synaptic transmission to depolarize the postsynaptic neurons, remove the Mg^{2+} block and induce activation of NMDARs (Malenka and Nicoll, 1999).

The voltage-sensitive block of NMDARs by Mg^{2+} and their high permeability to Ca^{2+} are determined by the amino acid residues in the pore lining domain of M2. The cation selectivity is dependent on a critical asparagine residue in the M2 segment of both the NR1 and NR2 subunits (Kuner et al., 1999). Other factors, such as protons,

polyamines and Zn^{2+} , also exert influence to the function of NMDARs. For instance, protons inhibit the activity of NMDARs through reducing the frequency of channel opening without changing the channel conductance (Traynelis et al., 1995). The IC_{50} for proton block is equivalent to pH 7.3, so even a little bit shift in pH from the physiological level will dramatically change the size of the NMDAR current (Traynelis and Cull-Candy, 1990). Zn^{2+} blocks the NMDAR current in a noncompetitive and voltage-independent manner, and increases the agonist-induced currents in the splice variants of homomeric NR1-1a and heteromeric NMDARs containing NR1-1a and NR1-1b at an EC_{50} of $0.5\mu M$ (Hollman et al., 1993). Taken together, the studies suggest that Zn^{2+} may act as a neuromodulator of NMDAR-mediated synaptic transmission, at least in some specific brain areas, since activation of glutamatergic neurons lead to the release of Zn^{2+} .

It is well documented that many of the important NMDARs properties are influenced by the subunit composition. NMDARs comprising merely homomeric NR1 subunits have been found in the oocytes (Nakanishi et al., 1998). In contrast, the NR2 family of NMDAR subunits is functionally inactive when expressed alone. NR1 and NR2 heteromers increase the NMDARs conductance 10- to 100-fold compared with homomeric NR1 receptors, indicating that NR2 subunits are indispensable for the high efficiency or function of NMDAR complexes (Liu and Zhang, 2000). As mentioned above, NR3 subunits are also involved in functional NMDARs when coexpressed with NR1 and NR2 subunits (Das et al., 1998). Each type of heteromeric receptors show an individual profile of functional properties, such as channel conductance, activation and

deactivation kinetics, agonist, antagonist and co-agonist sensitivities, and responses to channel blockers of Mg^{2+} and MK-801. For instance, NR1/NR2A receptors show a greater than 50-fold sensitivity to the inhibition of Zn^{2+} than NR1/NR2B receptors (Chen et al., 1997; Paoletti et al., 1997), and NR1/NR2A receptors are responsible for LTP induction whereas NR1/NR2B receptors are necessary for LTD induction (Liu et al., 2004).

1.2.4 Expression of NMDARs

Different NMDAR subunits show distinct developmental, brain-regional, cellular and subcellular expression patterns (Das et al., 1998; Hollmann & Heinemann 1994). NR1 subunits are expressed ubiquitously throughout the CNS at all developmental stages, as they are critical for NMDARs function.

The expressions of NR2 subunits are differentially regulated during development and in different brain areas, with particular NR2 subunits restricted to defined neuronal populations. In embryonic brain, only the NR2B and NR2D subunits are expressed (Watanabe et al., 1992). NR2B subunit is expressed widely in the embryonic brain, whereas NR2D subunit is found exclusively in the diencephalon and brainstem. During the first two weeks after birth the expression pattern of NR2 subunits changes dramatically. The NR2B subunits are replaced to a great extent by NR2A subunits (Chavis and Westbrook, 2001), which appear in the whole brain, and subsequently become restricted in the forebrain (Watanabe et al., 1992). NR2C subunits are found only in the cerebellum and NR2D subunits are almost completely abolished (Watanabe et al., 1992).

In the adult brain, NR2 subunits also show restricted distribution patterns. The NR2A subunits distribute widely, but have a higher level of expression in the cerebral cortex, the hippocampus and the cerebellar granule cells. The NR2B subunits are expressed selectively in the forebrain and higher levels of expression are found in the cerebral cortex, the hippocampus, the septum, the caudate-putamen, the olfactory bulb and the thalamus (Monyer et al., 1994). The NR2A and NR2B subunits also show different subcellular expression patterns. Although NR2A and NR2B are present both at synapses and extrasynaptic sites, studies have shown that NR2B subunits are expressed predominantly extrasynaptically (Dalby and Mody, 2003; Stocca and Vicini, 1998), whereas NR2A subunits show a higher content at synaptic than at extrasynaptic sites (Mohrmann et al., 2002; Thomas et al., 2006). The subcellular localization of NR2A- and NR2B-containing NMDARs and subsequently the route of Ca^{2+} influx into the neurons are known to activate distinct signaling pathways that can differ between synaptic and extrasynaptic sites. Ca^{2+} influx through extrasynaptic NMDARs activates large-conductance-type K^+ channels and mediate inhibition (Isaacson and Murphy, 2001), whereas Ca^{2+} influx via synaptic NMDARs activates small-conductance-type K^+ channels and mediate excitation (Faber et al., 2005; Ngo-Anh et al., 2005). Studies showed that the activation of extrasynaptic NR2B-containing NMDARs causes neurotoxicity (Hardingham et al., 2002) and is sufficient to induce LTD (Massey et al., 2004). The NR2C subunits are expressed predominantly in the granule cells of the cerebellum, with weak expression in the olfactory bulb and the thalamus. The NR2D subunits is only weakly expressed in the thalamus, the brainstem and the olfactory bulb (Monyer et al., 1994).

1.3 LTP and LTD

Communication between neurons in the mammalian brain is achieved through a process known as synaptic transmission. Some of the synapses have the ability to change their strength or efficacy of synaptic transmission, in a process referred to as synaptic plasticity. Synaptic plasticity is accomplished through changes in the transmitter release, and the properties, location and number of receptors in the synapse. Based on the lasting time, synaptic plasticity can be divided into short-term plasticity and long-term plasticity. It has been widely believed that short-lasting plasticity is achieved mainly through the presynaptic changes in the transmitter release (Klein, 1995), whereas both presynaptic changes (Corlew et al., 2007) and postsynaptic changes (Ahmadian et al., 2004) contribute to long-term lasting plasticity. Long-term potentiation (LTP) and long-term depression (LTD) of synaptic transmission are two of the best studied forms of synaptic plasticity in the mammalian brain and are widely believed underlying the cellular mechanism of learning and memory (Bliss and Collingridge, 1993).

1.3.1 LTP

LTP is the persistent increase in synaptic strength mediated by some specific neuronal activity. Since LTP was first discovered by Bliss and Lømo (1973), LTP has attracted the wide interests of neuroscientists because of its potential role in learning and memory. LTP has been investigated intensively in the rodent hippocampus, neocortex and amygdala, which are critical areas for memory storage.

To date, three distinct subtypes of LTP have been characterized. The best characterized subtype of LTP is NMDAR-dependent, which is sensitive to NMDAR antagonists and is frequently observed at the synapses of the pyramidal neurons in the CA1 region of the hippocampus. Another form of LTP is Ca²⁺-permeable AMPAR-dependent, which is insensitive to NMDAR blockers. There is another special form of LTP, hippocampal mossy fiber LTP, which is independent of both NMDAR and Ca²⁺-permeable AMPAR activation.

1.3.1.1 NMDAR-dependent LTP

NMDAR-dependent LTP can be achieved experimentally by either delivering high-frequency stimulation (HFS) or pairing postsynaptic depolarization with presynaptic stimulation (Gustafsson et al., 1987). Both approaches can make a coincidence of presynaptic glutamate release and the postsynaptic depolarization that subsequently activate NMDARs and allow extracellular Ca²⁺ influx through these activated NMDARs.

Accumulated evidence has shown that NR2A-containing NMDAR is associated with NMDAR-dependent LTP. Wang's group recently observed that the NR2A-specific antagonist NVP-AAM077 prevented LTP induction in the hippocampal slice, suggesting that activation of NR2A-containing NMDARs is required for the production of hippocampal LTP (Liu et al., 2004). This result was further confirmed by a more recent *in vivo* recording study using systemic injection of NVP-AAM077 (Fox et al., 2006).

NMDAR-dependent LTP is featured by the extracellular Ca^{2+} passing through NMDARs into postsynaptic neurons. There are probably other sources of Ca^{2+} responsible for the induction of NMDAR-dependent LTP, such as the Ca^{2+} passing through the voltage-gated Ca^{2+} channels (Grover and Teyler, 1990; Aniksztejn and Ben-Ari, 1991; Kullmann et al., 1992) and Ca^{2+} released from the intracellular compartments (Malenka et al., 1988).

The targets of endocytosed Ca^{2+} in the postsynaptic neuron after NMDARs activation include a few protein kinases, such as Ca^{2+} -calmodulin regulated kinase II (CaMKII), protein kinase A (PKA) and protein kinase C (PKC). Of these kinases, the role of CaMKII is the best characterized. Studies found that blockade of CaMKII with antagonists (Malenka et al., 1989; Malinow et al., 1989) or knockout of CaMKII in animals (Silva et al., 1992) can completely prevent the production of LTP. Conversely, introducing functional CaMKII into postsynaptic neurons significantly increases the synaptic efficacy (Lledo et al., 1995; Pettit et al., 1994). These findings combined with other observations support the idea that the activation of CaMKII following the NMDARs activation is necessary for the improved synaptic transmission. It is still unclear how activated PKA and PKC function in mediating synaptic excitation, but they may play regulatory roles in the LTP via phosphorylating NMDARs and/or AMPARs to increase their conductance (Boehm et al., 2006).

The detailed mechanism underlying LTP induction is controversial. Either presynaptic component or postsynaptic component or both may contribute to the LTP expression. The presynaptic machineries have a few options: (i) an increase in the probability of presynaptic glutamate-containing vesicles release; (ii) an increase in the

amount of glutamate contained in each vesicle released from the presynaptic membrane; (iii) a change in the spatial and/or temporal concentration profile of released glutamate so that NMDARs and/or AMPARs are exposed to a higher concentration of glutamate (Kullmann et al., 2000).

The postsynaptic components involved in LTP production are as follows: (i) a change in the properties of postsynaptic glutamate receptors so that the response of these receptors to glutamate is increased; (ii) an increase of the amount of postsynaptic glutamate receptors; (iii) an increase of the number of synapses (Kullmann et al., 2000).

These different phenomena may play roles at different times of LTP expression. Because LTP can be induced only in a little proportion of synapses, it is very difficult to measure the extracellular glutamate concentration, or to detect the response of glutamate receptors to the agonists (Bliss and Collingridge, 1993). Most information regarding the LTP expression mechanism is from examining the changes in signals elicited by activity in the same axons which were stimulated during LTP induction (Kullmann et al., 2000).

A major question against the simple increase in presynaptic transmitter release is that the short-term activity-dependent facilitation is not largely changed during LTP (McNaughton, 1982; Muller and Lynch, 1989; Manabe et al., 1993; Asztely et al., 1996). This finding is in contrast to the observation in pharmacological manipulations that alteration of presynaptic transmitter release almost uniformly changed the short-term facilitation (Manabe et al., 1993).

It is accepted by most neuroscientists that postsynaptic changes play a critical role in the LTP expression. Because LTP is associated with a relatively greater increase

in the potency of signal mediated by AMPARs than that mediated by NMDARs (Asztely et al., 1992; Kullmann, 1994), a so-called silent synapse hypothesis was proposed. According to this hypothesis, AMPARs are initially absent or nonfunctional at a population of synapses, but they can be recruited by the induction of LTP. That is, after LTP induction, AMPARs may undergo a relatively selective alteration in their conductance properties, or in the number of receptors available to detect presynaptic glutamate release.

Now it has been apparent that a major mechanism of the expression of LTP involves an increase of AMPARs in the postsynaptic plasma membrane, which is attributed to the regulated trafficking of AMPARs from intracellular compartments to the specific membrane region. Blocking this insertion of AMPARs to postsynaptic membrane can abrogate the NMDAR-induced LTP expression (Bredt and Nicoll, 2003; Song and Huganir 2002). Another process responsible for the induction of LTP is the modification of the biophysical properties of AMPARs themselves via direct phosphorylation (Lee et al., 2003; Soderling and Derkach, 2000). Activated CaMKII or PKC following NMDARs activation phosphorylates GluR1 subunits of AMPAR complex and increases their conductance efficacy. Therefore, the protein kinase CaMKII or PKC and the AMPAR subunit GluR1 play particularly important role in the induction of LTP (Boehm et al., 2006).

Whereas the above mentioned mechanisms are responsible for the initial phase of the increase in synaptic efficacy lasting no longer than one hour, actually LTP persists for hours, days, or even weeks. The mechanisms responsible for the so-called “late phase” of LTP have attracted great interests. It has been well characterized that the

late phase of LTP requires new protein synthesis and gene transcription (Abraham and Williams, 2003; Lynch, 2004). Varied protein kinases, including PKA, CaMKIV, and MAPK, are widely thought to convey the LTP-inducing activity to the nucleus. Upon activation, these protein kinases subsequently activate the key transcription factor cAMP response element binding (CREB), as well as the expression of the immediate early genes such as *zif268* (Abraham and Williams, 2003; Lynch, 2004). A hypothesis is that, during the synaptic activation resulting in LTP, a “synaptic tag” is generated that functions to capture or sequester plasticity-related proteins, which are required to stabilize the initial increase in synaptic strength (Frey and Morris, 1998). Steward et al (1998) showed that following LTP-induction stimulation Arc protein became locally enriched in the dendritic spines of the dentate gyrus. However, to date, nothing is known about the identity of the “synaptic tag” (Malenka and Bear, 2004).

Another possibility for the longer-term maintenance of LTP is that those synapses at which LTP has occurred may undergo structural remodeling. Morphological changes have been reported, including the growth of new dendritic spines, enlargement of preexisting spines and associated postsynaptic densities (PSDs), and the splitting of single PSDs and spine into two functional synapses (Abraham and Williams, 2003; Yuste and Bonhoeffer, 2001). Studies demonstrated that LTP was accompanied by expansion of the postsynaptic density and enlargement of dendritic spines (Okamoto et al., 2004; Matsuzaki et al., 2004). Interestingly, this increase in spine size only occurred transiently in the large, more mature and mushroom-shaped spines, but was persistent in smaller spines (Malenka and Bear, 2004). The actin cytoskeleton is greatly enriched in dendritic spines (Matus, 2000), and it is thought that

the polymerization of actin plays a key role in the structural alterations of dendritic spines in maintaining LTP, and actin filament reorganization is likewise associated with the shrinkage of spines during LTD (Okamoto et al., 2004; Wang et al., 2007). Consistent with this notion, inhibitors of actin cytoskeleton polymerization impaired LTP (Kim and Lisman, 1999; Krucker et al., 2000). Furthermore, LTP *in vivo* is also accompanied by a long-lasting increase in F-actin content within spines, which appears to involve the inhibition of actin depolymerization (Fukazawa et al., 2003).

Studies have also shown that LTP involves the addition of “scaffolding proteins” that act as placeholders for AMPARs at the post synaptic densities (Malinow and Malenka, 2002; Shi et al., 2001), and this greatly contributes to the synaptic growth. Dendritic synthesis of the key proteins including CaMKII and AMPAR subunits may also play critical roles in the structural changes during LTP maintenance (Ju et al., 2004; Steward and Schuman, 2001).

1.3.1.2 Ca²⁺-permeable AMPAR-dependent LTP

Ca²⁺-permeable AMPAR-dependent LTP is a special subtype of activity-dependent LTP that can be induced in the presence of NMDAR antagonists. This form of LTP has been observed at synapses in some nuclei of amygdala (Mahanty and Sah, 1998) and dorsal horn of the spinal cord (Gu et al., 1996). A very interesting functional characteristic of LTP triggered by Ca²⁺-permeable AMPARs is that Ca²⁺ influx is likely to be maximal when presynaptic glutamate release coincides with postsynaptic hyperpolarization. This is because GluR2-deficient receptors characteristically show an inwardly rectifying current-voltage relationship. Thus at a network level, plasticity may

be expected to follow different rules regarding the coincidence of pre- and postsynaptic activity from those of NMDAR-dependent LTP. Although synapses with Ca^{2+} -permeable AMPARs are also seen in hippocampal interneurons, high frequency stimulation induces LTD rather than LTP (Laezza et al., 1999). This finding implies that there are additional factors determining the direction of synaptic strength change.

1.3.1.3 Mossy fiber LTP

Mossy fiber LTP was first observed at hippocampal mossy fiber synapses on CA3 pyramidal neurons (Nicoll and Malenka, 1995). Later on, mechanistically similar forms of LTP were also seen at corticothalamic synapses (Castro-Alamancos and Calcagnoto, 1999) and cerebellar parallel fiber synapses (Linden, 1997; Salin et al., 1996). The induction of this form of LTP does not require the coincidence of presynaptic stimulation and postsynaptic depolarization. Application of glutamate receptor antagonists or blockers fail to prevent the induction of mossy fiber LTP, suggesting that AMPARs, NMDARs and kainite receptors are not involved in this kind of LTP (Kullmann et al., 2000). Studies showed that the expression of mossy fiber LTP is presynaptic: high-frequency stimulation of presynaptic afferent fibers triggers a significant increase of cyclic AMP (cAMP) in presynaptic terminals, which leads to a persistent enhancement of transmitter release (Nicoll and Malenka, 1995). In addition, another study suggests that the activation of presynaptic kainite receptors by endogenous glutamate plays an important role in triggering mossy fiber LTP (Schmitz et al., 2003; Contractor et al., 2001).

Recent research has focused on the detailed mechanism underlying mossy fiber

LTP. Pharmacological and genetic manipulations suggest that a rise in presynaptic calcium induces mossy fiber LTP, in part by activation of calcium-stimulated adenylyl cyclases (ACs) and subsequently increased cAMP and activation of PKA (Wang et al., 2003; Nicoll and Malenka, 1995). At first the prime presynaptic candidates of PKA substrates that play critical roles in mossy fiber LTP were synapsins I and II, which are robustly phosphorylated by PKA (Sudhof et al., 1989). However, normal mossy fiber LTP could be induced in synapsin I- or II-deficient mice (Spillane et al., 1995), and hippocampal LTP could still be elicited in synapsin I and II double-knockout mice (Rosahl et al., 1995), indicating that these two proteins are dispensable in mossy fiber LTP. In contrast, mossy fiber LTP is absent in mice lacking the synaptic vesicle protein Rab3A (Castillo et al., 1997), which binds to two different PKA substrates, the synaptic vesicle-associated rabphilin and the major active zone constituent RIM1a (Sudhof, 2004). Follow-up studies showed that rabphilin knockout mice still exhibited normal mossy fiber LTP (Schlüter et al., 1999), whereas mossy fiber LTP was absent in RIM1a knockout mice (Castillo et al., 2002). Thus, mossy fiber LTP appears to require the interaction of Rab3A and RIM1a, both of which are proteins function at the interface of synaptic vesicles and the active zone (Malenka and Bear, 2004).

1.3.2 LTD

A different form of synaptic plasticity, LTD refers to the persistent decrease in synaptic efficacy triggered by some specific neuronal activity. LTD can be experimentally induced by prolonged low-frequency stimulation (LFS) (Dudek and

Bear, 1992). At least two subtypes of LTD, which are pharmacologically distinct, have been identified.

1.3.2.1 NMDAR-dependent LTD

NMDAR-dependent LTD involves the Ca^{2+} influx via activated NMDARs. Studies also have shown that NR2B-containing NMDARs are necessary for the induction of this type of LTD. Liu et al (2004) showed that NR2B subunit-selective antagonists Ro25-6981 and ifenprodil completely abolished the induction of LTD in the hippocampal slice preparation, suggesting that activation of NR2B-containing NMDAR is required for inducing hippocampal LTD. These results were further confirmed by a more recent *in vivo* recording study using systemic injection of Ro25-6981 (Fox et al., 2006). Recently Duffy et al. also reported that NMDAR co-agonist D-serine significantly enhanced LTD in mice hippocampal slice, which was completely reversed by NR2B specific antagonist Ro25-6981, suggesting that NR2B is necessary for hippocampal LTD production (Duffy et al., 2007).

Similar to LTP, Ca^{2+} influx in the postsynaptic dendritic spine triggers NMDARs-dependent LTD. This has attracted wide interest in how the Ca^{2+} influx through NMDARs can induce both LTP and LTD, two opposite directions of synaptic strength change. One hypothesis is that the direction and extent of the change in synaptic strength is determined by the amplitude of the Ca^{2+} influx. In principle, a brief but large Ca^{2+} influx activates protein kinases such as CaMKII, PKC (Lee et al., 2000), PKA (Fourcaudot et al., 2008), and phosphatidylinositol-3-kinase (PI3K) (Opaza et al., 2003), subsequently causing phosphorylation of downstream targets and leading to LTP;

whereas a more prolonged but modest Ca^{2+} influx activates phosphatases including protein phosphatase 1 (PP1) (Morishita et al., 2001), protein phosphatase 2B (calcineurin) (Fujiwara et al., 2007), and phosphatase and tension homolog located on chromosome 10 (PTEN) (Wang et al., 2005), thereby dephosphorylating some related proteins and inducing LTD (Bear, 1995; Yang et al., 1999).

Redundant studies have been performed to investigate the mechanism of LTD expression. One finding is that LTP and LTD are expressed as a consequence of bidirectional changes in postsynaptic AMPARs phosphorylation, which happens at the C-terminus of the GluR1 subunits. Lee et al (2000) found that the expression of LTP was associated with phosphorylation of ser-831, which is a substrate of protein kinase CaMKII and PKC, without a change in ser-845, a PKA substrate. In the same study, LTD was found conversely to be associated with selective dephosphorylation of ser-845 but not ser-831. Because dephosphorylation of ser-845 decreases the open probability of AMPAR channel, it is reasonable that it takes partial responsibility of LTD expression (Banke et al., 2000). In a recent study, mice with a “knockin” GluR1 mutant which harbors alanine substitutions for serines 845 and 831 showed a NMDAR-dependent LTD deficiency in CA1 region of the hippocampus (Lee et al., 2003).

Actually the most intensively explored mechanism of LTD expression is the internalization of AMPARs. To date, how LFS rapidly induces the endocytosis of the AMPARs from the postsynaptic membrane surface still remains elusive, but accumulated evidence showed that this process depends on the interactions between the C-terminal tails of AMPAR subunits and intracellular proteins such as clathrin. A number of studies have revealed that the AMPAR GluR2 subunit is a key regulator of

the AMPAR endocytosis that initiates LTD. A synthesized peptide GluR2-3Y derived from rat GluR2 carboxyl tail, which was reported to specifically block regulated AMPAR endocytosis, could completely prevent LFS-induced LTD (Ahmadian et al., 2004; Wang et al., 2004).

Given the essential role of AMPARs in mediating LTD, the question arises how the stable numbers of synaptic AMPARs are maintained. It seems that a class of “scaffolding proteins” that act as place holders for AMPARs at the synapse are necessary. PSD-95 has been expected to be a kind of scaffolding protein. PSD-95 is attached to the postsynaptic membrane by PDZ interactions with the C-terminal tails of NMDAR subunits (Sheng, 2001). PSD-95 also binds to stargazin, which induces the insertion of AMPARs into the postsynaptic membrane (Schnell et al., 2002). Via this interaction with stargazin, PSD-95 could potentially act as a docking site for AMPARs at the synapse. Another interfering peptide NR2B9c, derived from the intracellular C-terminal of NR2B, was recently found to be able to block the first step of the induction of hippocampal NMDAR-dependent LTD (Wang et al, preliminary observation).

1.3.2.2 mGluR-dependent LTD

The best characterized and described form of mGluR (metabotropic glutamate receptor)-dependent LTD occurs in the cerebellar cortex at parallel fiber synapses to Purkinje cell, when they are stimulated in conjunction with the climbing fiber input (Ito, 1989). Activation of the climbing fiber causes a robust influx of Ca^{2+} and this significant rise of intracellular Ca^{2+} subsequently induces LTD. However, a key event that distinguishes active from inactive parallel fiber synapses, and that is required to

trigger LTD, is the activation of postsynaptic group 1 metabolic GluRs (Linden et al., 1991).

The mechanism of mGluR-dependent LTD has been studied in detail in cultured Purkinje neurons, and it has several common features with the NMDAR-dependent LTD discussed above. The data suggest that LTD-inducing stimuli also lead to clathrin-dependent internalization of AMPARs (Wang and Linden, 2000). Similarly, in cultured hippocampal neurons, activation of group 1 mGluRs can also trigger the rapid removal of AMPARs from postsynaptic membrane (Snyder et al., 2001).

1.4 Amygdala

1.4.1 General anatomy of amygdala

The amygdala is an almond-shaped structure located in the deep medial temporal lobe. This structure has attracted continued interest because of its central role in emotional processing. Structurally the amygdala is diverse and comprises about thirteen nuclei. These nuclei can be further divided into three groups: 1) the deep or basolateral group, which includes the lateral nucleus, the basal nucleus, and accessory basal nucleus; 2) the superficial or cortical-like group, which includes the cortical nucleus and nucleus of the lateral olfactory tract; and 3) the centromedial group, which is composed of medial and central nucleus. Finally, there is another group of nuclei that can not be easily put into any of these three groups and are listed separately. These nuclei include the intercalated cell masses and the amygdalohippocampal area (Sah et al., 2003). (Figure 1.3)

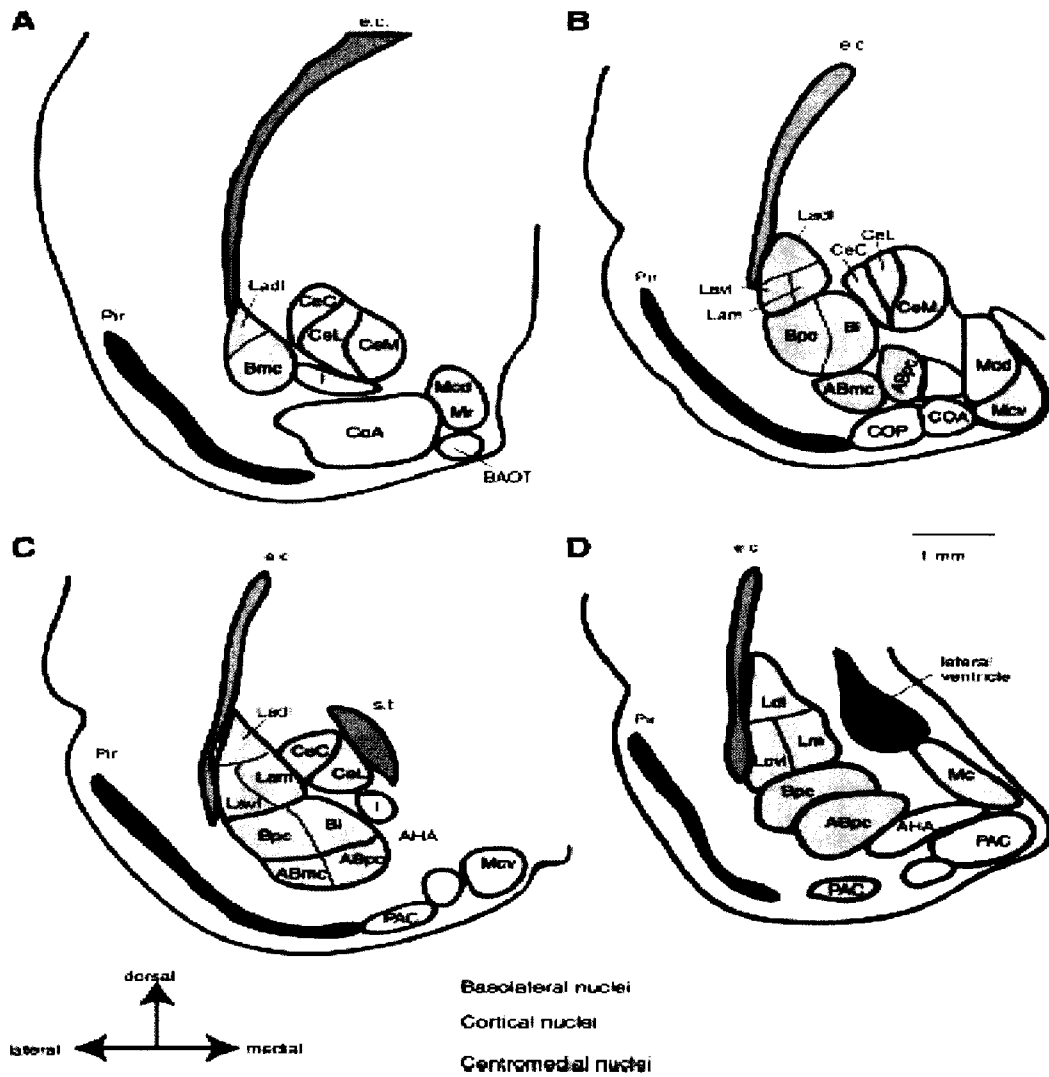


Figure 1.3 Classification of the amygdala complex. Also showing their relative positions. (Cited from Sah et al., 2003)

1.4.2 Connections of the amygdala

Neuroanatomical studies have revealed the detailed connection of the amygdala. Each amygdaloid nucleus receives input projections from multiple but different brain areas, and the amygdala also send projections widely to both cortical and subcortical brain regions (Pitkanen, 2000).

1.4.2.1 Afferent connections of the amygdala

According to the input information, the afferent projections to the amygdala can be separated into those arising from cortical and thalamic brain structures, and those arising from the hypothalamus and brain stem. Cortical and thalamic inputs supply information from sensory regions and structures related with memory systems, whereas those hypothalamic and brain stem inputs arise brain areas involved in behaviour and autonomic systems (Sah et al., 2003). The amygdala receives inputs from all modalities, including olfactory, somatosensory, gustatory and visceral, auditory, and visual modalities through the afferent pathways from the cortex and thalamic structures.

There are a few sources of polymodal sensory information to the amygdala. These brain regions include prefrontal cortex, perirhinal cortex, and hippocampus. The prefrontal cortex is a major source of cortical projections to the amygdala (Sah et al, 2003). Information from sensory modalities usually converges in the prefrontal cortical regions (Ray and Price, 1992). The basal nuclei are the main target of afferent inputs from the prefrontal cortex. There are also reciprocal and strong connections between the amygdala and those brain areas, including the perirhinal cortex, the entorhinal cortex, the parahippocampal cortex and the hippocampus, which are associated with

learning and memory formation (Milner et al., 1998). The lateral amygdala is the major target of the perirhinal cortex, which also sends projections to the basal and cortical like nuclei (Shi and Cassell, 1999). In contrast, the projections arising from the entorhinal cortex are sent to most regions of the amygdala (McDonald and Mascagni, 1997). The subicular area is the main source of the inputs from the hippocampus to the amygdala and most projections are sent to the basal nucleus (Canteras and Swanson, 1992). As well, the brain stem regions including the midbrain, pons and medulla also send a large amount of projections to their major target, the central nucleus, while other parts of the amygdala receive few or no innervation from these regions (Pitkanen, 2000).

1.4.2.2 Efferent connections of the amygdala

When receiving afferent inputs from the cortical, hypothalamic and brain stem areas, the amygdala also sends widespread projections to and innervate these important brain regions (Sah et al, 2003).

Generally, the cortical and basolateral nuclei send a few projections to the cortical sensory regions. The basolateral and medial nuclei of the amygdala project to those areas in the frontal cortex, and the cortical nuclei also send projections back to the olfactory cortex. The basolateral group nuclei, including lateral, basal and accessory basal nuclei, send substantial projection to the medial temporal lobe memory system including hippocampus and perirhinal cortex, and also send a large number of projections to the nucleus accumbens (Pitkanen, 2000). The medial part of the centromedial group substantially projects to the hypothalamus, bed nucleus of the stria terminalis and a few nuclei in the brain stem (Dong et al., 2001; Veening et al., 1984).

The medial and capsular nuclei of the central group also send projections to the lateral structures of the hypothalamus (Petrovich et al., 2001), which subsequently projects to the brain stem and spinal cord (Swanson and Kuypers, 1980). The lateral subdivision of the central nucleus and the cortical nuclei send projections to the lateral hypothalamus as well. The detailed efferent connectivity of different nuclei is summarized as Figure 1.4.

1.4.2.3 Intra-amygdala connections

Except for those large quantities of afferent inputs from many brain areas to the amygdala and projections to other brain regions, there are also extensive intranuclear and internuclear connectivity within the amygdala. For example, the sensory information entering the amygdala through the basolateral nuclei, is processed locally, then follows predominantly lateral nuclei to the centromedial nuclei, which serve as an output station to some other brain structures (Pitkanen et al., 1997).

It has been found that there are extensive retrocaudal and interdivisional connections within the lateral amygdala. The presence of the lateral to medial intranuclear connections within the lateral amygdala suggests that the medial subdivision might be the site for integration of sensory information with assessments of past experience (Sah et al., 2003). The lateral amygdala also extensively projects to the basal and accessory basal nuclei and the capsular part of the central nucleus (Pitkanen et al., 1995), which have many internuclear and intranuclear connections as well. The largest projection from the basal nuclei is to the medial part of the central nucleus

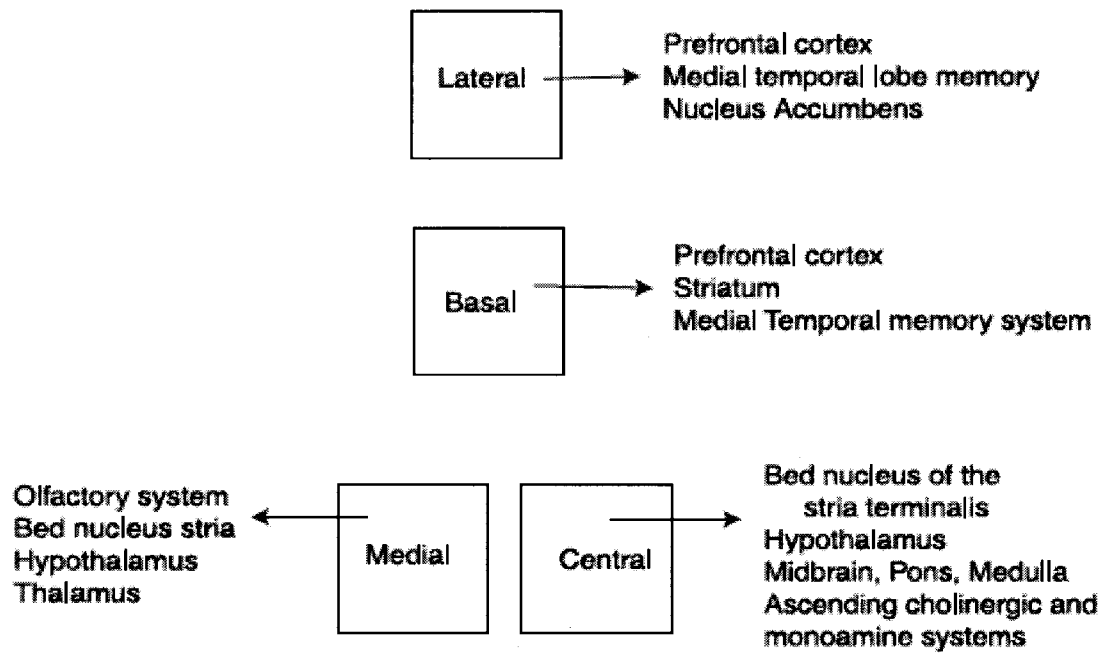


Figure 1.4 Summary of the efferent projections of the amygdala complex. (Cited from Sah et al., 2003)

(Savander et al., 1996). The accessory basal nucleus sends a large amount of afferents to the lateral amygdala and the central and medial nuclei (Savander et al., 1996).

Interestingly, the central nucleus, which receives many inputs from all the other parts of the amygdala, sends very few projections back to those nuclei. Within the central nucleus of the centromedial group there are extensive intradivisional and interdivisional connections. The central division of the central nucleus sends a large quantity of projections out of the amygdala, and also sends a moderate number of projections to the capsular subdivision (Jolkkonen and Pitkanen, 1998).

The detailed intra-amygdala connectivity is summarized as Figure 1.5.

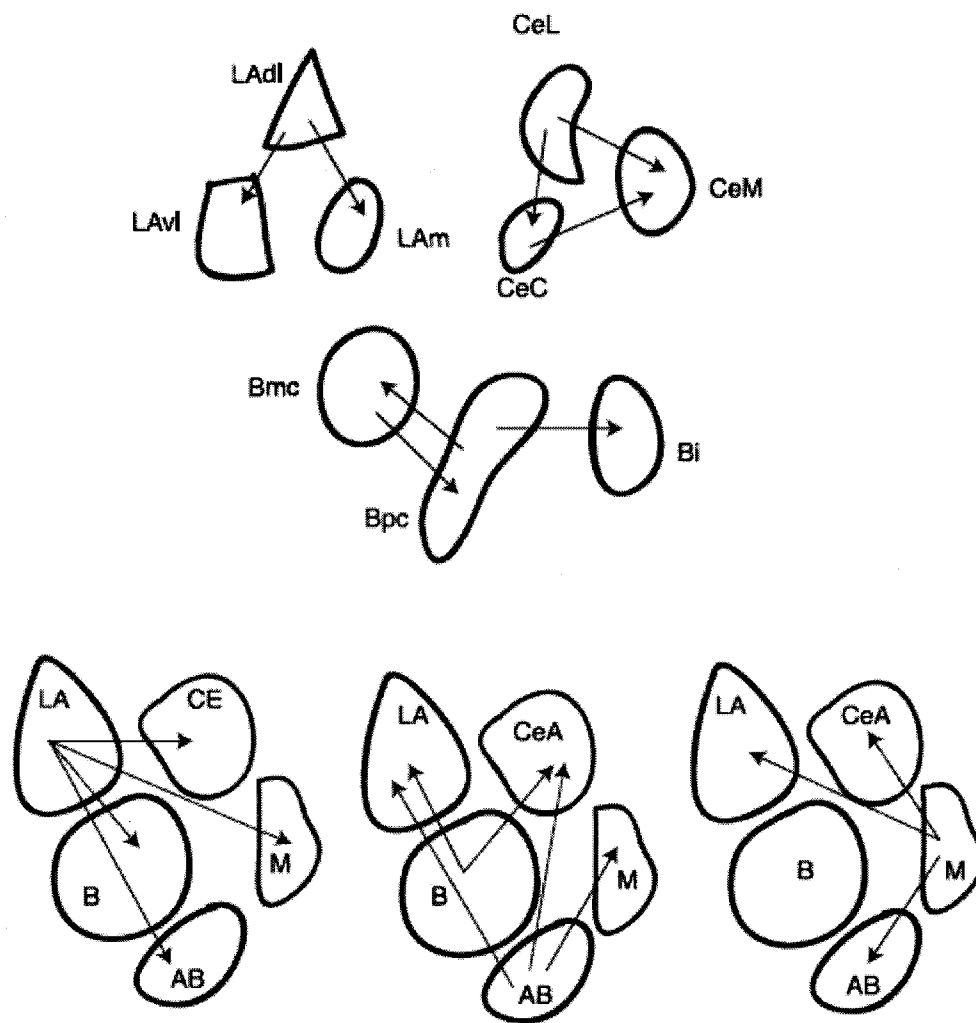


Figure 1.5 Summary of the intra-amygdala and internucleus connectivities within the amygdala complex. (Cited from Sah et al., 2003)

1.4.3 Function of the amygdala

The amygdala has been the major focus for studying the mechanisms underlying fear and anxiety in animals. As mentioned above, anatomically the amygdala receives information from all sensory modalities via reciprocal connections with the cerebral cortex, the thalamus, the hippocampus and other subcortical structures, and plays a central role in assessing the emotional significance of this information, modulating memory formation, and orchestrating the behavioural responses (LeDoux, 1992; Davis, 1994; Goldstein et al., 1996; Fanselow & Gale, 2003; McDonald, 2003; Sah et al., 2003). Via efferent pathways from the central nucleus of the amygdala to the hypothalamus and brain stem, the amygdala can also activate neuroendocrine and autonomic responses during stressful situations (Davis, 1992, 1994; Habib et al., 2001). Thus, the function of the amygdala is most intimately related to the neurobiological mechanisms that underlie emotional behaviour. It is not surprising therefore that emotional disorders are often associated with pathophysiological changes in the amygdala (Abercrombie et al., 1998; Chen et al., 2005). For example, a characteristic feature of anxiety disorders, such as PTSD, is an enhancement of neuronal excitability in the amygdala (Rauch et al., 2000; Villarreal & King, 2001). Recent functional magnetic resonance imaging (fMRI) studies in humans showed that increased amygdala activation was associated with several affective states including fear and anxiety (Furmark et al., 1997; Adolphs et al., 1998; Tillfors et al., 2001; Critchley et al., 2002). It was also reported that NMDAR current and proteins were downregulated in the amygdala during the maintenance of fear memory, suggesting that NMDARs in the amygdala plays a critical role in fear conditioning, a model of emotional learning and

cue-induced anxiety (Zinebi et al., 2003).

1.5 NMDARs are involved in emotional behaviours, especially in anxiety

There is growing evidence in support of an important role of glutamate neurotransmission in the mediation of emotion and in the pathophysiology of mood and anxiety disorders. It has been reported that NMDARs, a well-characterized subtype of glutamate receptors, are involved in stress responses (Del Acro et al., 2001; Ho et al., 2000; Okano et al., 1995), while anxiety is the primary response to stress (Lesse, 1982; Pinel, 2002). For example, infusion of a low dose of NMDAR antagonist AP5 into the ventral hippocampus produced a significant anxiolytic effect in the EPM test (Häckl and Carobrez, 2007). Although AP5 is a nonselective antagonist to NR2 subunits, NR2A-containing NMDARs are more sensitive to a low dose of AP5 than NR2B (Liu et al., 2004).

Recently NR2C-2B mutant mice have been generated, in which the NR2C genes were completely replaced by NR2B genes expression throughout the brain. This NR2C-2B mutant was used to examine whether an NMDAR subunit exchange in juvenile mice would affect emotional behaviours. The results showed that the NR2C-2B mice exhibited decreased dwell time in open arms in the EPM. Thus, the replacement of subunit NR2C by NR2B in NR2C-2B mice was suggested to increase anxiety-related behaviours (De Souza Silva et al., 2006). However, this study also showed that both NR1 and NR2A proteins were dramatically increased in the hippocampus of the NR2C-2B mice. It is unknown, therefore, the increased anxiety in the NR2C-2B transgenic

mice resulted from the increase of NR2B or NR2A or both.

A recent study showed that NR2A knockout mice exhibited decreased anxiety-like behaviour relative to wild-type littermates across multiple tests including EPM, light–dark exploration test, and novel open field test (Boyce-Rustay & Holmes, 2006), suggesting that genetic inactivation of NR2A subunits decreases anxiety in this model. These findings are in agreement with the most recent study that after chronic exposure to a neurotoxic benzo[a]pyrene (B[a]P) the expression of NR2A gene in the hippocampus, the hypothalamus, the cerebellum and the temporal cortex was significantly decreased, and meanwhile the mice showed lower level of anxiety than controls in the EPM task (Grova et al., 2008).

In summary, the above evidence suggests that inactivation of NR2A-containing NMDARs, genetically or with antagonists, produces significant anxiolytic effect in the innate anxiety model such as EPM. Although the role of NR2B subunits in innate anxiety remains unknown, it is possible that NR2B plays a role that is opposite to that played by NR2A, because activation of NR2A- and NR2B-containing NMDARs induces LTP and LTD, respectively, as discussed above.

1.6 Research Project

1.6.1 Summary of reviewed literature

A series of evidence have shown that NMDARs, especially the NR2A- and NR2B-containing NMDARs, are critically involved in unconditioned anxiety process.

As well, there is accumulated evidence showing that NR2A- and NR2B-containing NMDARs are critically involved in LTP and LTD induction, respectively, in

many brain regions such as the hippocampus and the amygdala. These two forms of long-term neural plasticity are widely thought to underlie many important brain functions, such as learning, memory and drug addiction.

In the past few decades, many related studies have revealed that the amygdala is an essential component of the pathway that assigns emotional significance and produce appropriate behavioural response to the external stimuli (LeDoux, 2000). Specifically the NMDARs expressed in the amygdala play a very important role in the emotional behaviours.

1.6.2 Hypothesis

Based on the above lines of evidence we **hypothesize that inactivation of NR2A- and NR2B-containing NMDARs alleviates and aggravates innate anxiety via blockade of amygdaloid LTP and LTD, respectively.**

1.6.3 Objectives

The purpose of the present study is to investigate the possible opposite roles in innate anxiety of NR2A- and NR2B-containing NMDARs and the subsequent LTP and LTD they mediate. Therefore, we will address the following objectives:

Objective 1 is to study the alleviatory effects of NR2A-containing NMDAR antagonist on anxiety.

Objective 2 is to study the aggravating effects of NR2B-containing NMDAR antagonists on anxiety.

Objective 3 is to study the aggravating effects of blocking LTD on anxiety.

2. MATERIALS AND METHODS

2.1 Subjects

Long-Evans rats were purchased from Charles River Laboratories (St-Constant, Quebec), weighing 200-225g on arrival. Animals for Elevated Plus-Maze test were doubly housed whereas those for Novelty Suppressed Feeding test were housed individually in standard plastic cages (45 × 25 ×20 cm). Lighting for the housing room was maintained on a 12-h light/dark cycle (lights on at 07:00h and off at 19:00h automatically) and the climate was controlled at 22±1°C, relative humidity at 60%. Animals were given free access to animal chow and water *ad libitum* throughout the experiment. All experiments were conducted in accordance with the rules of the Canadian Council of Animal Care, and were approved by the animal care committee of the University of Ottawa Institute of Mental Health Research.

All rats were allowed at least five days of acclimation to the new housing environment before behaviour tests or surgery.

2.2 Drugs

NR2A selective antagonist, NVP-AAM077, was a generous gift from Dr. Paolo Paganetti, Novartis Pharma AG (Basel, Switzerland). NR2B specific antagonists, Ro25-6981 and ifenprodil, and diazepam were purchased from Sigma-Aldrich MO (St. Louise, USA). To block NMDAR-dependent LTD, we employed two peptides: 1) GluR2-3Y, a synthetic peptide derived from the rat GluR2 carboxyl tail (Tyr-Lys-Glu-Gly-Tyr-Asn-Val-Tyr-Gly), can specifically block the clathrin-coated regulated but not constitutive AMPAR endocytosis and hence block the LTD induction, without effect on

LTP (Ahmadian et al., 2004; Wang et al., 2004); 2) NR2B9c, a peptide comprising the nine C-terminal amino acids of NR2B (Lys-Leu-Ser-Ser-Ile-Glu-Ser-Asp-Val), can specifically block the protein-protein interaction between NR2B and PSD95 (Aarts et al., 2002) and thus NR2B-induced LTD. GluR2-3Y and NR2B9c 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) (Schwarze et al., 1999). Tat-GluR2-3Y and the scrambled peptide were kindly supplied by Dr. Yu Tian Wang, Brain Research Center of the University of British Columbia (Vancouver, Canada). Ro25-6981 (6mg/kg), ifenprodil (5mg/kg), and NVP-AAM077 (1.2mg/kg or 2.4mg/kg) were dissolved in 0.9% saline at 2mg/ml, 5mg/ml, and 0.8mg/ml, respectively. Tat-GluR2-3Y (3 μ mol/kg) and the scrambled peptide and Tat-NR2B9c (3 μ mol/kg) were dissolved in 0.9% saline as well at 3 μ mol/ml. Diazepam (1mg/kg or 2.5mg/kg) was dissolved in a mixed solution of DMSO, TWEEN 80 and saline (1 : 1 : 18). The prepared Ro25-6981 solution was diluted to 100 μ M and used for intra-amygdala infusion (1 μ l/injection/cannula). All solutions were freshly made before injection to animals. Those respective vehicles were used for control injection.

2.3 Apparatus

The Elevated Plus-Maze was constructed with hard wood and consisted of two opposite open arms 50 cm \times 10 cm (surrounded by a 1 cm high Plexiglas ledge to prevent rats falling off the maze), and two opposite enclosed arms, 50 cm \times 10 cm \times 40 cm, set up 50 cm above the floor. The junction area of the four arms (central platform)

measured 10 cm × 10 cm. The upper surfaces of all arms and the inner surface of walls were covered with clear Plexiglas.

2.4 Procedures

2.4.1 Locomotor activity test

Thirty minutes after completion of injection in the housing room, animals were transported to the behavioural test laboratory and locomotor activity was examined. Each rat was put in the center of the activity box placed in a sound-attenuating room. The 12 × 12 horizontal infrared beams recording mood was used and the total horizontal movements in 5 min was recorded.

2.4.2 Elevated Plus-Maze

The elevated plus-maze test was performed as described previously (Handley and Mithani, 1984; Holmes et al., 2003). Immediately after the locomotor activity test, rat was removed from the activity box and placed onto the central platform of the plus-maze facing an open arm, and allowed to explore the apparatus for 5 min. Time spent in and entries into open arms and enclosed arms were recorded. An arm entry was recorded when all four paws of the rat were in the arm. Measures of anxiety include **dwell ratio** [$\text{open time}/(\text{open time} + \text{enclosed time}) \times 100\%$], and **entry ratio** [$\text{open entries}/\text{enclosed entries} \times 100\%$], with lower values indicating higher levels of anxiety. Immediately after the 5 minutes testing, rats were removed from the maze and put back to the home cage and returned to the housing room. After each trial, the maze and the activity box were thoroughly cleaned with paper towel dipped in 70% alcohol solution,

and evaporated in the air to dry before the next trial.

2.4.3 Modified Novelty-Suppressed Feeding Test

All dishes and holders were set up a few days before testing and the scoring sheets, timers and scale were also prepared in advance. All dishes and holders were numbered and they would be used with the same rats each day. All animals were allowed at least five days of acclimation to the new housing environment before testing. On day 1 of home cage testing, about 5 grams of Graham Crumbs was weighed and added into each dish. The food dishes within the holders were placed into the middle front of the cages as quickly and quietly as possible, and the timer was started immediately. The rats were observed closely for 15 minutes. The latency for rats to consume the Graham Crumbs and the amount consumed by each rat within the 15 minutes were recorded. On the following testing days the amount of Graham Crumbs added into the dishes were increased according to the amount that the rats had eaten the day before. After each day's test all dishes and holders were thoroughly cleaned and dried in the air. During the home cage test period no cage change was made. After approximately 10 days all rats should have baseline levels of grams and latency. At least 3 days of consistent amount of food consumed and 2 days when most of the rats started to eat within one minute were required before the drug effect was tested. For the novel cage test, rats were transported to new cages without bedding immediately after injection, and the feeding test was conducted in a novel behavioural testing room other than the housing room.

2.4.4 Surgery

Rats were deeply anesthetized with continuous inhalation of isoflurane (5% for induction and 2.5% for maintenance). Once unresponsive to tailpinch, rats were placed in a stereotaxic frame instrument and stereotaxically and bilaterally implanted with 22 gauge guide cannulae at the following coordinates taken from Paxinos and Watson 1982: A/P -3.1mm, L + 5.3 mm, D/V -7.7 mm. Four jeweler screws were attached to the skull, and the entire assembly was cemented in place using Cranioplastic Powder (Plastic Products). Stainless steel wires (i.e., stylets) were inserted into the guide cannula to maintain potency. The tip of each wire was extended about 1 mm past the end of the guide cannula. For pain control, rats received intraperitoneal (i.p.) injection of Temgesic (0.03mg/kg) twice daily for three days after surgery. Rats were allowed a seven-day recovery period after surgery and were handled gently every day in the following five days. Bilateral intra-BLA infusions (0.5µl/min, 1µl/amygdala) were made through 28-gauge injection cannulae that were attached by polyethylene tubing to a microsyringe. Injection cannulae were left in place for 2 min after the infusion was completed to allow diffusion of the drugs or vehicle, after which the stylets were placed back into the guide cannulae. 30 min later, rats were tested in the EPM.

2.4.5 Histology

Following the completion of EPM test, animals receiving the guide cannulae implantation were euthanized with intraperitoneal (i.p.) injection of 40% (wt/vol) chloral hydrate overdose and were perfused intracardially with 0.01M phosphate-buffered saline (PBS) followed by 4% (wt/vol) paraformaldehyde. Then rats were

decapitated and brains were removed and immersed in 4% paraformaldehyde solution and incubated at 4°C for at least 24 hours, and were subsequently transferred into 30% (wt/vol) sucrose-PBS solution and incubated for at least three days prior to sectioning. Brains were flash frozen with dry ice, and were sectioned with a sliding microtome into series of 40- μ m-thick frontal sections, which then were mounted on gelatin-subbed glass slides and processed for standard Cresyl Violet staining to verify the exact locations of the guide cannulae in the BLA. Only those rats with at least one cannula (of the bilateral pair) correctly positioned (10 of 16 rats in treatment group and 11 of 15 in control group) were used in the data analysis.

2.4.6 Data Analysis

All data were expressed as mean \pm SEM and analyzed with independent-sample T-test. $P < 0.05$ was considered to be significant throughout. All statistical calculations were carried out using SPSS, version 9.0 software.

3. RESULTS

3.1 Pilot experiments to determine the preferred rat strains for innate anxiety models

During the course of performing pilot experiment with the EPM test, we observed a great variance of both dwell ratio and entry ratio among normal Wistar rats. Then, we tested Long-Evans rats and obtained consistent results by two independent experimenters (Data not shown). Thus, Long-Evans rats have been used in all studies proposed here.

3.2 The EPM and NSF models are appropriate in evaluating the effectiveness of anxiolytic drugs

To validate the animal models of anxiety (EPM and NSF) that were used in this project, we employed the well documented anxiolytic drug, diazepam, as a reference. In the EPM, diazepam (i.p., 2.5mg/kg) significantly increased both the dwell ratio (Figure 3.1A) and entry ratio (Figure 3.1B), but produced no significant effect on locomotor activity (Figure 3.1C). Similarly, in the NSF, diazepam (i.p., 1mg/kg) significantly decreased the latency (Figure 3.2A) and increased the amount consumed (Figure 3.2B) compared to the vehicle control group. These results support the effectiveness of the EPM and NSF models in measuring innate anxiety levels of rats following anxiolytic or anxiogenic drug interference.

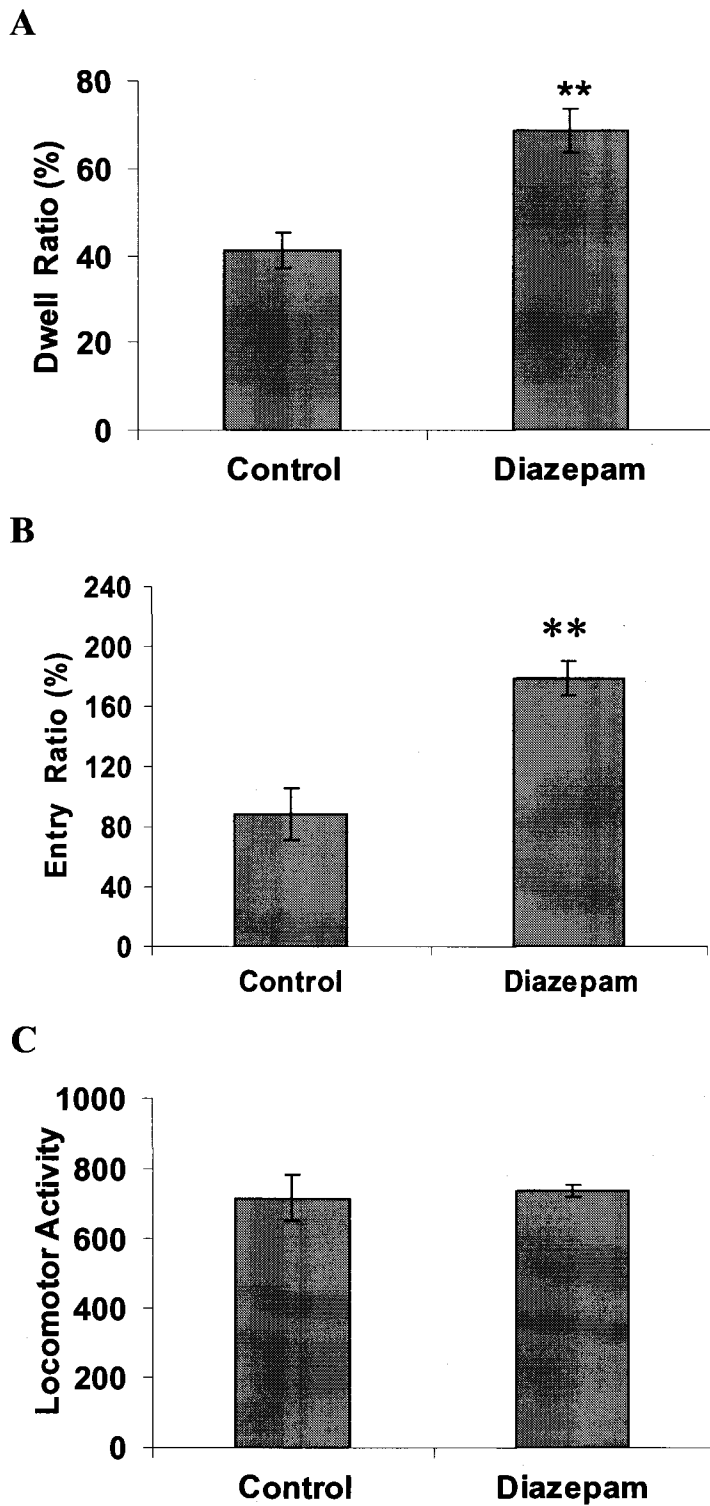


Figure 3.1 The systemic administration of diazepam (2.5mg/kg) produced significant anxiolytic effect in the elevated plus-maze. Both the dwell ratio (A, $**P < 0.01$) and the entry ratio (B, $**P < 0.01$) were significantly increased by the pre-treatment of diazepam ($n = 9$), as compared to the vehicle controls ($n = 8$). But the locomotor activity (C) was not significantly affected by diazepam ($P = 0.78$).

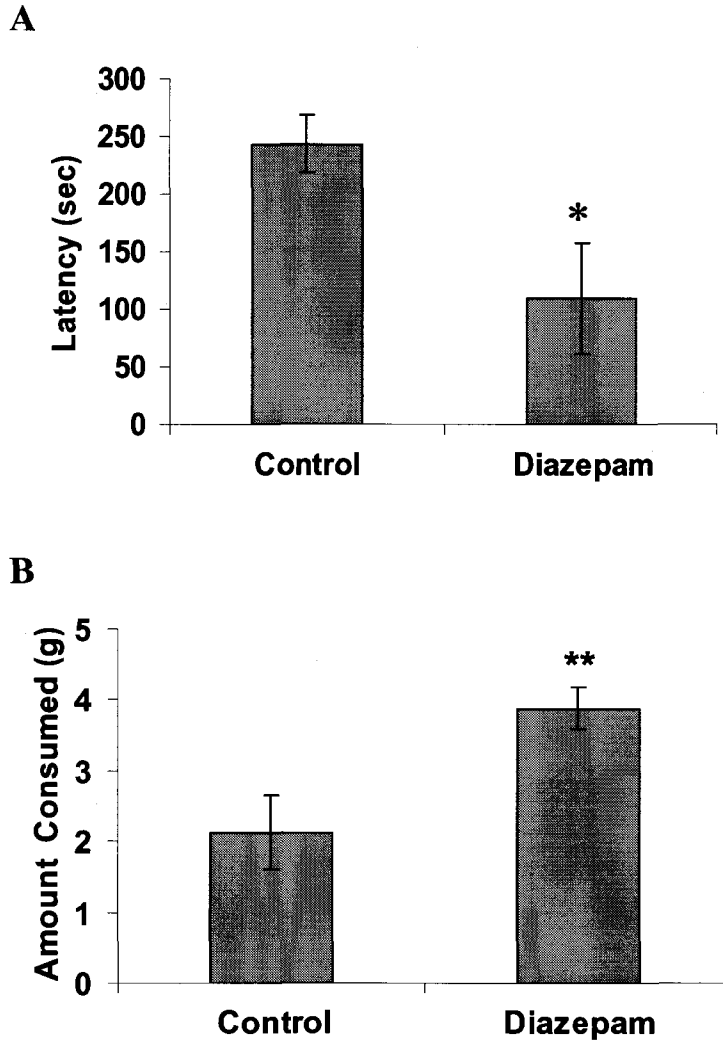


Figure 3.2 The i.p. application of diazepam (1mg/kg) significantly affected the latency (A) and the amount consumed by rats (B) in the novel environment suppressed feeding test conducted in an unfamiliar environment. * ($P < 0.05$) Decreased latency or ** ($P < 0.01$) increased amount consumed compared to vehicle controls on the same day novelty test.

3.3 NR2A antagonist NVP-AAM077 did not affect rat innate anxiety levels in EPM and NSF models

An i.p. injection of NVP-AAM077 (2.4mg/kg) did not significantly affect either dwell ratio or entry ratio in the EPM test between experimental and control rats receiving NVP-AAM077 and vehicle injections, respectively (Figure 3.3), suggesting NVP-AAM077 produced no significant effect on innate anxiety.

Then we examined a lower dose (1.2mg/kg) of NVP-AAM077 in the NSF. We also found that the latency and the amount consumed were not significantly affected either in the home cages (Figure 3.4) or in novel cages (without bedding and in a novel environment) (Figure 3.5). But the novel environment did significantly prolonged the consuming latency and reduced the amount eaten by rats (Data not shown), suggesting that novelty did produce a significant anxiogenic effect to these animals.

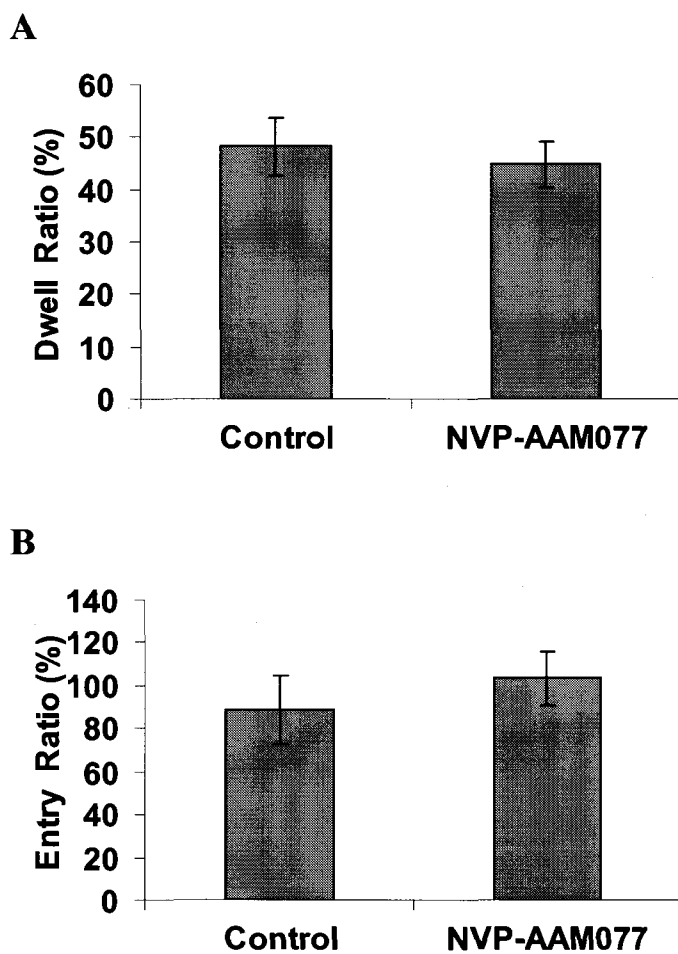


Figure 3.3 The systemic (i.p.) administration of NVP-AAM077 (2.4mg/kg) produced no significant effect on the anxiety-like behaviour in the elevated plus-maze. Neither the dwell ratio (A, $P = 0.63$) nor the entry ratio (B, $P = 0.48$) was significantly affected by the pre-treatment of NVP-AAM077 ($n = 8$), as compared to the vehicle controls ($n = 8$).

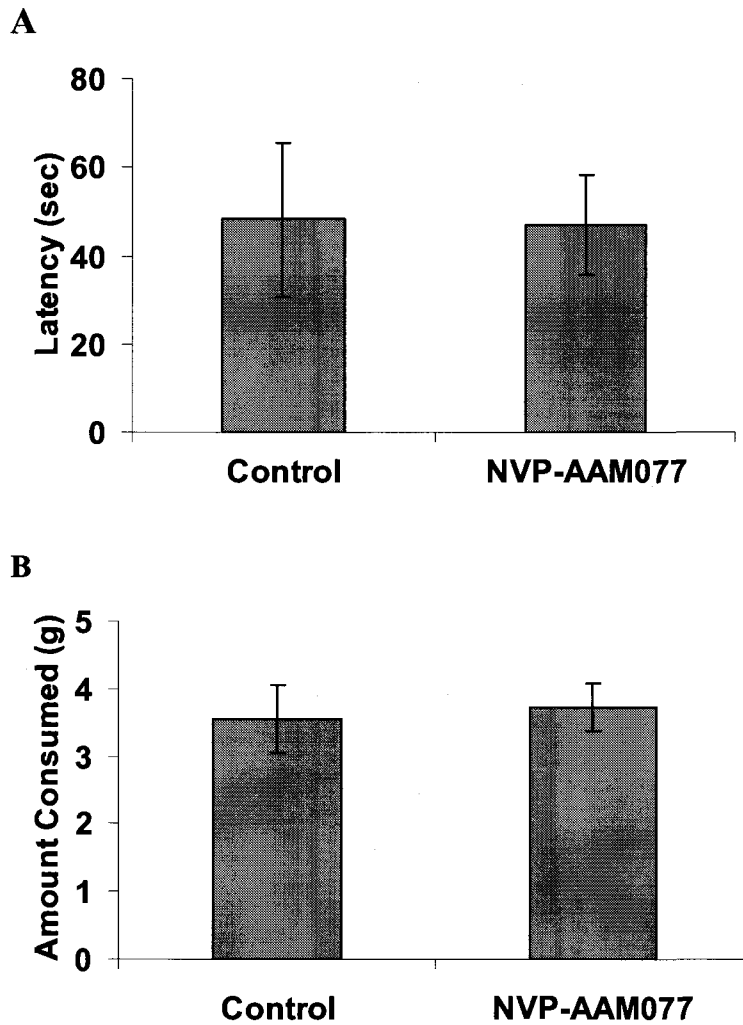
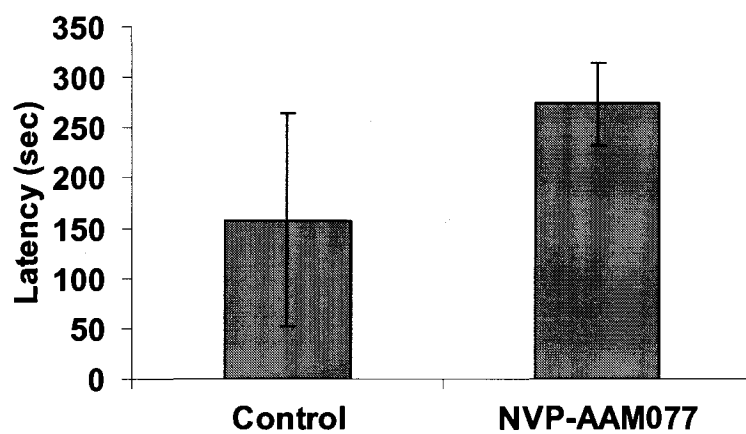


Figure 3.4 The i.p. application of NVP-AAM077 (1.2mg/kg) did not significantly affect the latency (A) and the amount consumed (B) of novel environment suppressed feeding test in home cage test.

A



B

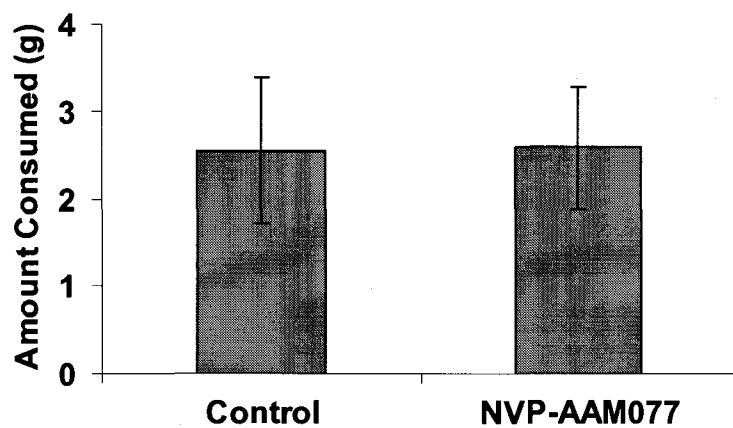


Figure 3.5 The i.p. application of NVP-AAM077 (1.2mg/kg) did not significantly affect the latency and the amount consumed by rats of novel environment suppressed feeding test in an unfamiliar environment.

3.4 NR2B antagonists Ro25-6981 and ifenprodil aggravated innate anxiety in EPM and NSF models

An i.p. injection of the NR2B-selective antagonist Ro25-6981 (6mg/kg) significantly decreased the entry ratio and dwell ratio in the EPM test (Figure 3.6A, B), but produced no significant effects on locomotor activity (Figure 3.6C). Similarly, in the NSF test, Ro25-6981 significantly prolonged the consuming latency (Figure 3.7A), and another NR2B-selective antagonist ifenprodil (5mg/kg) significantly decreased the amount consumed by experimental subjects (Figure 3.8B). These results suggest that blockade of NR2B-containing NMDARs aggravates innate anxiety in rats.

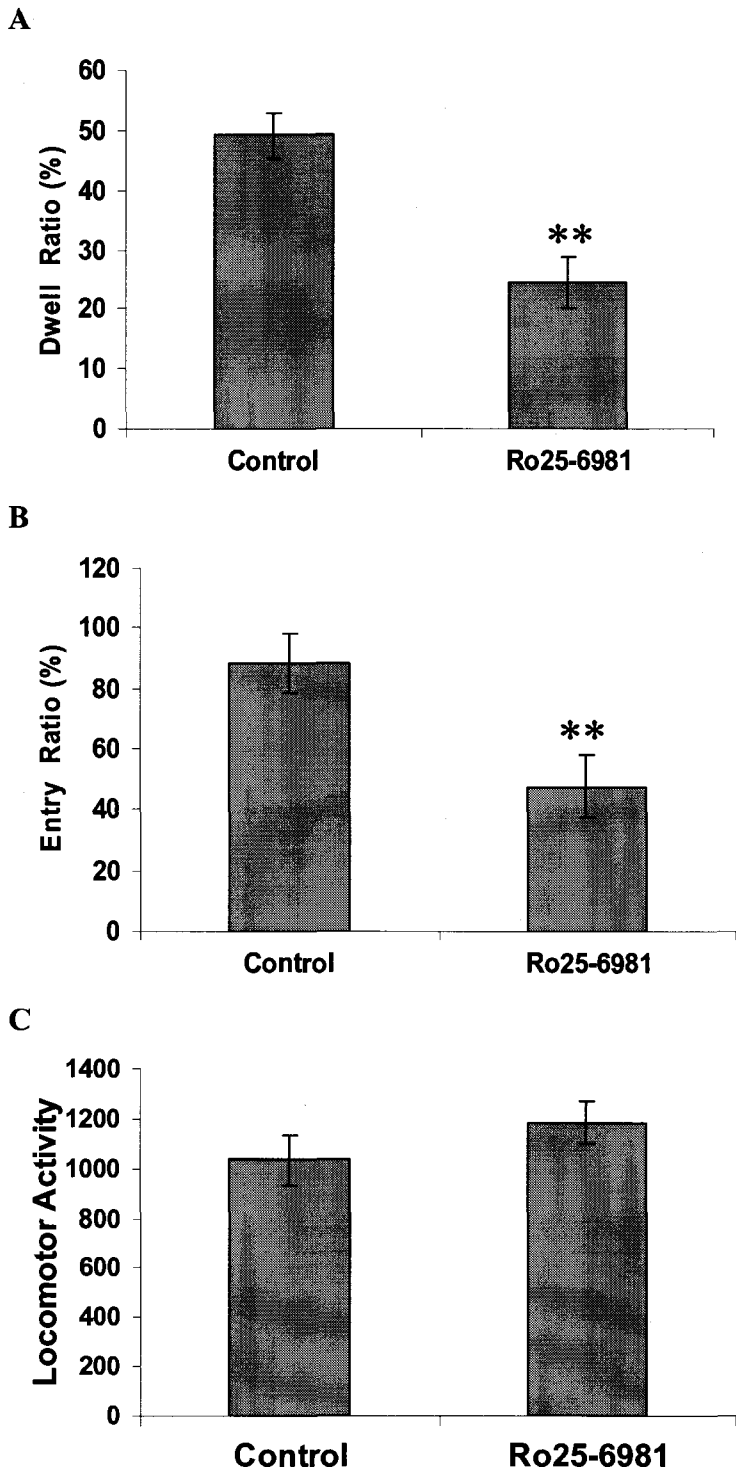


Figure 3.6 The i.p. administration of Ro25-6981 (6mg/kg) produced significant anxiogenic effect in the elevated plus-maze. Both the dwell ratio (A, $**P < 0.01$) and entry ratio (B, $**P < 0.01$) were significantly reduced by the pre-treatment of Ro25-6981 ($n = 10$), as compared to the vehicle controls ($n = 10$). But the locomotor activity was not significantly affected by Ro25-6981 ($P = 0.4$).

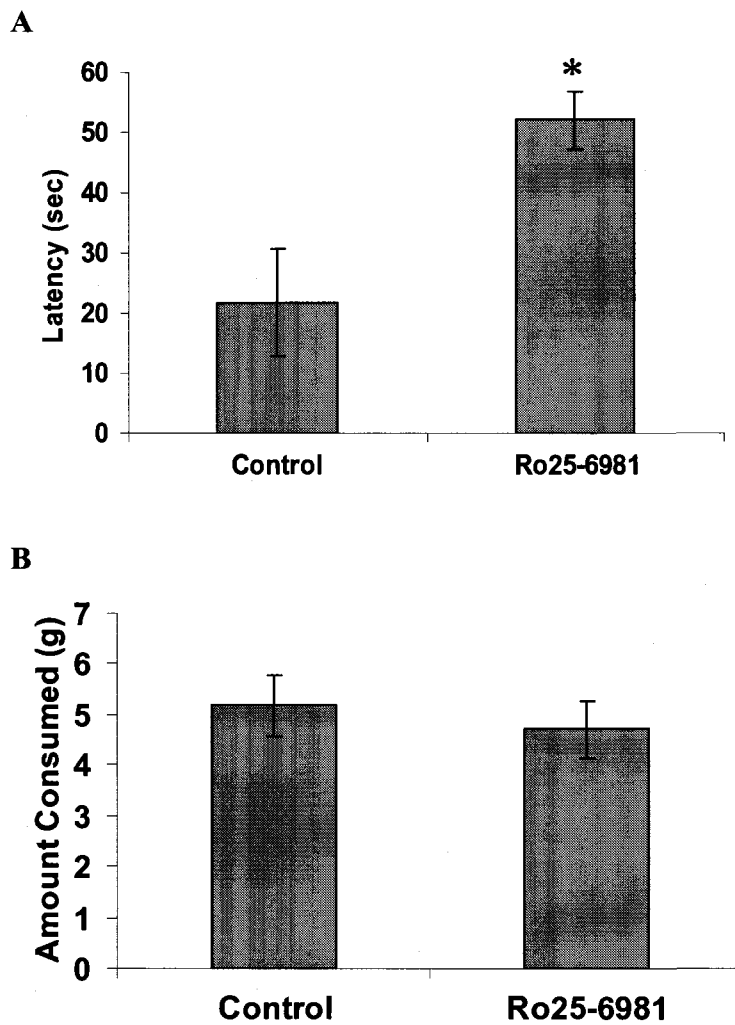


Figure 3.7 The i.p. application of Ro25-6981 (6mg/kg) significantly increased the consuming latency (A) (* $P < 0.05$), but the amount consumed (B) was not significantly affected ($n = 7-8$, $P = 0.59$). Tests were conducted in home cages.

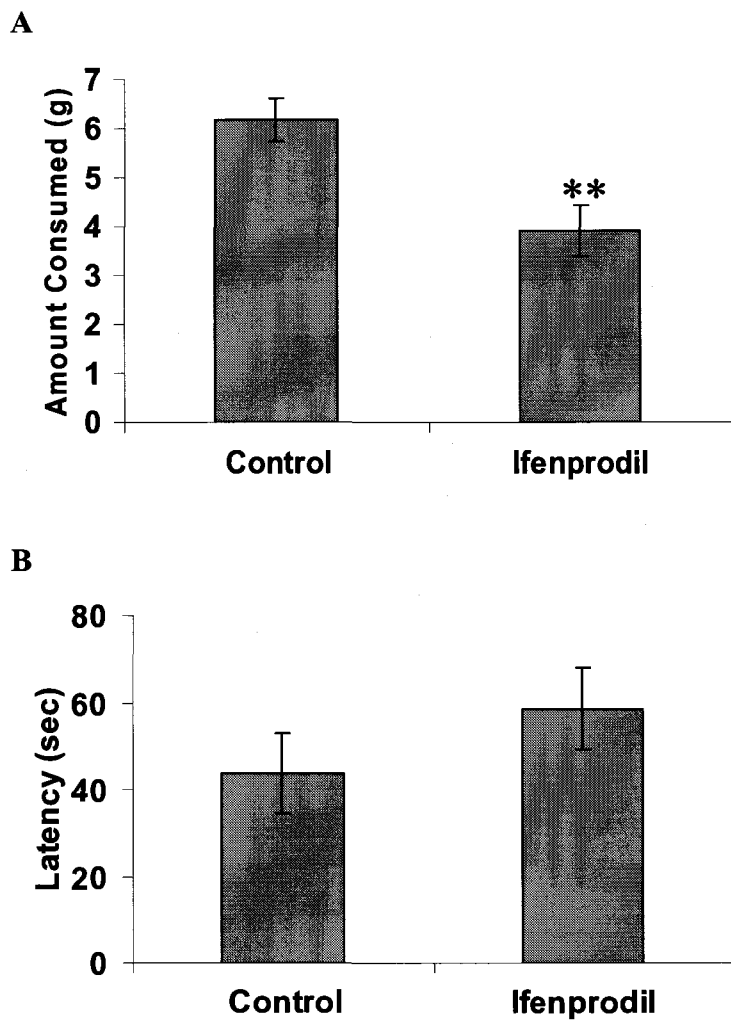


Figure 3.8 The i.p. application of ifenprodil (5mg/kg) significantly decreased the amount consumed (B) (** $P < 0.01$), but the latency (A) was not significantly affected ($n = 8$, $P = 0.28$). Tests were conducted in home cages.

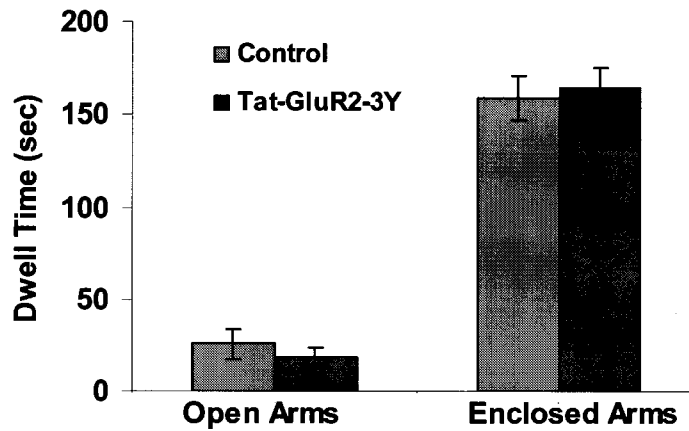
3.5 GluR2- and NR2B-derived LTD-blocking peptides failed to relieve innate anxiety in rats

As described above, NMDAR-dependent LTD may be involved in anxiety disorders. In addition, Yu Tian Wang's laboratory in the University of British Columbia discovered that activation of NR2A- and NR2B-containing NMDAR was required for the production of amygdaloid LTP and LTD, respectively (Yu et al., 2008). Therefore, we further examined whether the LTD-blocking peptides, Tat-GluR2-3Y, which is derived from GluR2, and Tat-NR2B9c, a NR2B-derived peptide, could enhance anxiety in rats.

In a pilot experiment we gave an intravenous (i.v.) injection of LTD-blocking peptide Tat-GluR2-3Y or scrambled control peptide (1.5 μ mol/kg) under a brief isoflurane anesthesia. Both groups of 10 rats each spent most time in the enclosed arms in the EPM (Figure 3.9A), but showed normal locomotor activity (Figure 3.9B), implying that the isoflurane anesthesia itself may produce anxiogenic effects.

Then, we conducted the NSF test in the home cages after i.p. administration of Tat-GluR2-3Y (3 μ mol/kg) without anesthesia. We found that both consuming latency and the amount consumed by rats were not significantly affected by Tat-GluR2-3Y (Figure 3.10). Similarly, another LTD-blocking peptide Tat-NR2B9c (3 μ mol/kg) produced no significant effects on the latency or the amount consumed by rats (Figure 3.11).

A



B

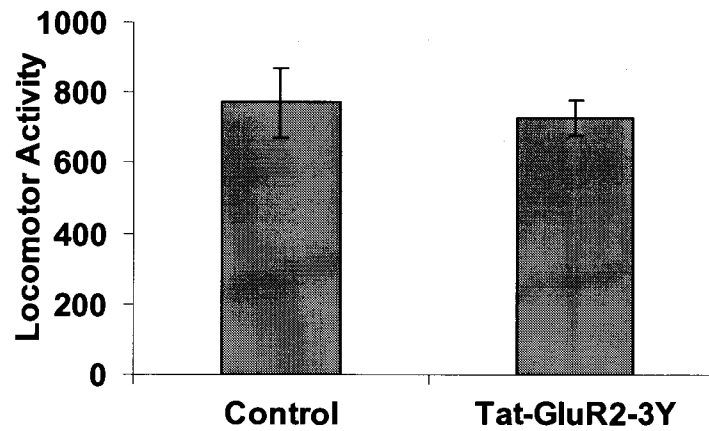


Figure 3.9 i.v. injection of Tat-GluR2-3Y or scrambled control peptide (1.5 μ mol/kg) under a brief isoflurane anesthesia made 10 rats in each group spend most time in the enclosed arms in the EPM (A), but did not significantly affect the locomotor activity (B).

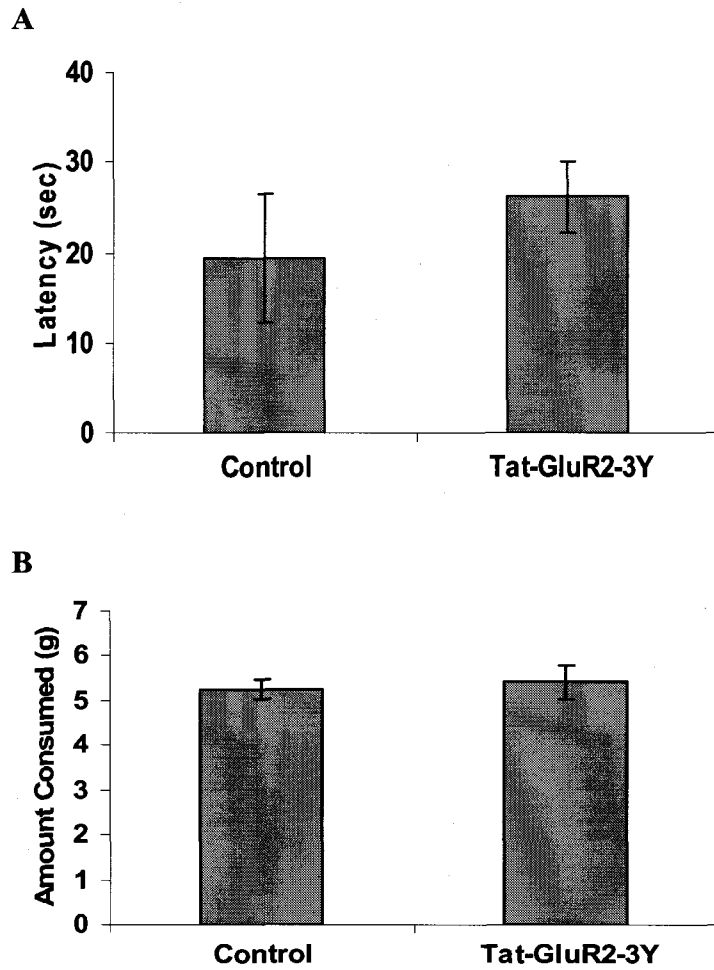


Figure 3.10 i.p. application of Tat-GluR2-3Y (3 μmol/kg) did not significantly affect the latency (A) ($P = 0.43$) and the amount consumed by rats (B) ($P = 0.72$) in novel environment suppressed feeding test performed in home cages.

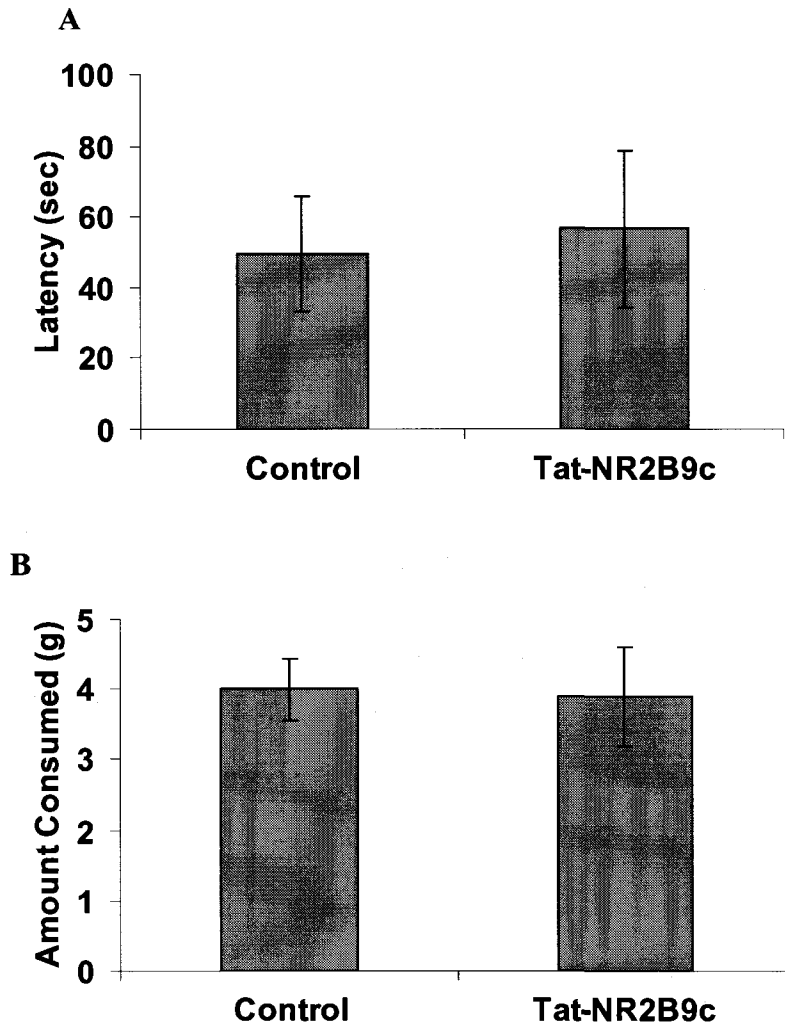


Figure 3.11 i.p. application of Tat-NR2B9c (3 μmol/kg) did not significantly affect the latency (A) ($P = 0.48$) and the amount consumed by rats (B) ($P = 0.39$) in novel environment suppressed feeding test performed in home cages.

3.6 Intra-amygdala injection of NR2B antagonists Ro25-6981 produced an anxiolytic outcome opposite to its systemic administration

The intra-amygdala infusion of Ro25-6981 (100 μ M, 1 μ l/cannula) significantly increased the dwell ratio (Figure 3.12A) compared to vehicle group, but did not significantly affect the entry ratio (Figure 3.12B) or the locomotor activity (Figure 12C). A trend of increase of entry ratio was produced but did not reach the significant level of difference. This is totally opposite to the above results of systemic injection of Ro25-6981 (Figure 3.4 & Figure 3.5).

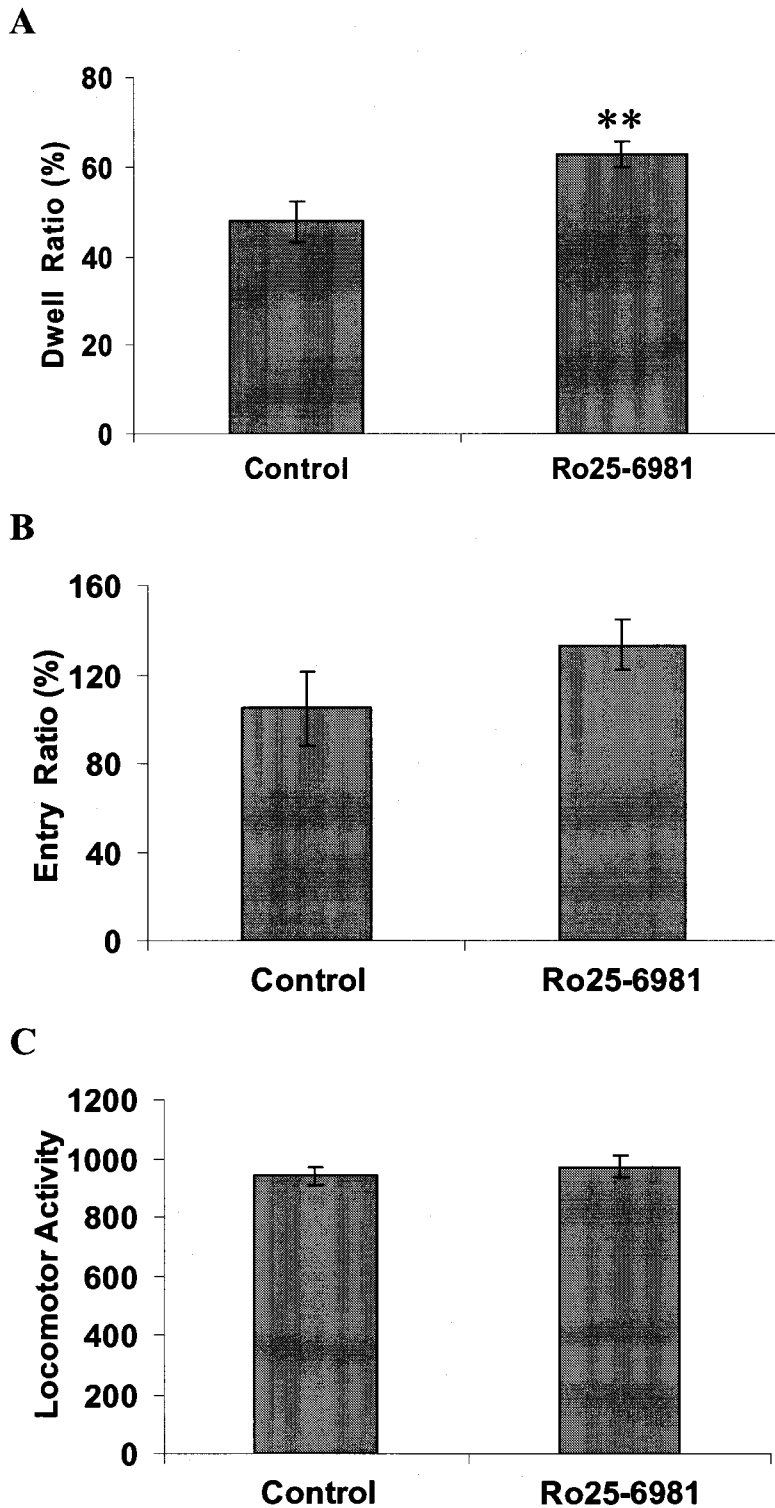


Figure 3.12 The intra-amygdala infusion of Ro25-6981 (100 μ M, 1 μ l/cannula) produced significant anxiolytic effect in the EPM. The dwell ratio (A, $**P < 0.01$) were significantly increased by the pre-treatment of Ro25-6981 ($n = 10$), as compared to the vehicle controls ($n = 11$). But the entry ratio (B, $P = 0.17$) and the locomotor activity (C, $P = 0.56$) were not significantly affected by Ro25-6981.

4 . DISSCUSSION

NMDARs are the major type of excitatory glutamatergic receptors in the CNS. Among the four NR2 subunits, NR2A and NR2B are predominant in the whole brain during development and in the adults, especially in the hippocampus and the amygdala. Both NR2A and NR2B have attracted wide attention for their critical roles in the normal brain functions and dysfunctions, such as synaptic transmission, neuronal plasticity, and learning and memory. It is established that NR2A and NR2B subunits confer to NMDARs distinct electrophysiological and signaling properties (Barria and Malinow, 2005; Vissel et al., 2002).

Due to the lack of specific antagonist or agonist for NR2A, a precise understanding of the physiological role of NR2A had been greatly limited in the past decades until a relatively selective NR2A antagonist, NVP-AAM077, was developed a few years ago. Nevertheless, the selectivity of NVP-AAM077 is still in debate. Some studies have argued that NVP-AAM077 has only 10-fold higher selectivity for NR2A than NR2B (Frizelle et al., 2006). The insufficiency of NVP-AAM077 in discriminating NR2A from NR2B was proved by a recent study showing that the higher dose of NVP-AAM077 (2.4mg/kg, i.p.) completely blocked the induction of hippocampal LTD *in vivo*, reminiscent of the effect of an NR2B-specific antagonist, while the lower dose (1.2mg/kg, i.p.) did not affect LTD (Fox et al, 2006). The above results also suggested that NVP-AAM077, after i.p. injection, could be effective and be functioning in some brain areas, especially in the CA1 region of the hippocampus. In our laboratory, similar amygdaloid LTP and LTD recording will be conducted after i.p. administration of NVP-

AAM077 to further prove that NVP-AAM077 is effective in the amygdala after systemic injection. In this study, both the higher dose and the lower dose of NVP-AAM077 was i.p. administered in rats, followed by examination of the innate anxiety levels in EPM and NSF tests. However, neither model revealed altered anxiety levels after NVP-AAM077 treatment. While the intact anxiety levels in rats receiving high dose NVP-AAM077 may reflect the mixed effects of this antagonist on NR2A and NR2B, our data suggest that systemic blockade of NR2A-containing NMDARs is insufficient to affect innate anxiety in rat EPM and NSF models. Employing fear-conditioning test, the animal model of learned anxiety, a recent study showed that the NR2A antagonist NVP-AAM077 disrupted fear conditioning when infused into the amygdala prior to conditioning session and also significantly disrupted fear expression when infused prior to testing session (Walker and Davis, 2008). Because in our study employing animal models of innate anxiety, the systemic administration of NVP-AAM077 did not affect the innate anxiety level of the animals, we did not further examine the effect of intra-amygdala infusion of NVP-AAM077. After the opposite effects on innate anxiety of i.p. and intra-BLA infusion of NR2B antagonist Ro25-6981 were observed, there appears the possibility that intra-BLA infusion of NVP-AAM077 may produce a different effect on anxiety from the i.p. administration. But Fox et al (2006) showed that local infusion of NVP-AAM077 to the hippocampus blocked both NMDAR-dependent LTP and LTD, suggesting that NVP-AAM077 is lack of selectivity when administered to specific brain regions. In summary, the above lines of evidence support the idea that NR2A-containing NMDARs play an important role in learned anxiety without significant contributions to innate anxiety.

As described in the introduction section, the role of NMDAR NR2B subunit in innate anxiety remains elusive. We demonstrated here that a systemic injection of the NR2B-containing NMDAR antagonists Ro25-6981 and ifenprodil produced an unexpected anxiogenic effect in both EPM and NSF models. In the NSF task, Ro25-6981 and ifenprodil significantly affected the latency and the amount consumed, respectively, but neither one influenced both measures. This may reflect their distinct effect on different patterns of innate anxiety. Anxiety-related behaviours are dependent on the specific characteristics of the test situation. Anxiety may be associated with stressor-related pathologies, combined with other psychological states (such as depression) (Furukawa et al., 2001), or may reflect the characteristics of the state or trait (Belzung and Griebel, 2001). Thus it is not surprising that anxiety response may involve diverse neuronal pathways differentially activated by the stressor/medication (Davis, 1998). Since the locomotor activity of the rats was not significantly altered by drug administration of Ro25-6981 (in the EPM test), the anxiogenic effect of Ro25-6981 can not be explained by altered locomotor function.

However, intra-amygdala injection of NR2B antagonists Ro25-6981 produced an anxiolytic effect, which is in contrast to the anxiogenic effect produced by systemic administration of Ro25-6981 or ifenprodil. These opposite effects on rat innate anxiety levels could be produced by the possibility that NR2B-containing NMDARs in the amygdala and other brain regions may make opposite contributions to innate anxiety. Häckl and Carobrez (2007) showed that ventral infusion of a low dose of NMDAR antagonist AP5 produced an anxiolytic-like effect in EPM test. Another study (Liu et al., 2004) proved that NR2A, but not NR2B, is more sensitive to low dose of AP5, since

LTP, rather than LTD, is prevented by low concentration of AP5. Therefore there is a possibility that the NR2B-containing NMDARs in the ventral hippocampus have an opposite role in innate anxiety to those in the basolateral amygdala. The effect of intra-amygdala infusion of NR2B-containing NMDARs has also been studied recently with fear-conditioning test, the animal model of learned anxiety. Walker and Davis (2008) revealed that infusion of the NR2B specific antagonist CP101,606 into the amygdala prior to conditioning sessions significantly disrupted fear conditioning, whereas infusion of the CP101,606 into the amygdala prior to testing did not influence fear-potentiated startle, suggesting intra-amygdala infusion of NR2B-containing NMDAR antagonist disrupted fear conditioning but not fear expression. Therefore, NR2B-containing NMDARs make significant contributions to both innate and learned anxiety.

While our results strongly suggest the involvement of NR2B-containing NMDARs in anxiogenic mechanism of innate anxiety, recent studies have also showed that activation of NR2B-containing NMDARs in the hippocampus and the amygdala was able to induce LTD as described in the introduction section. Therefore, we further asked whether the involvement of NR2B-containing NMDARs in innate anxiety requires LTD induction. To answer this important question, we employed the two LTD-blocking peptides GluR2-3Y and NR2B9c. GluR2-3Y is derived from rat GluR2 carboxyl tail and can specifically block LTD induction featured by AMPARs endocytosis, the last step of the induction of hippocampal LTD (Brebner et al., 2005; Fox et al., 2006; Wong et al., 2007). A more recent study also showed that bath application of GluR2-3Y completely blocked LTD induction in the amygdaloid slice preparation (Yu et al., 2008), suggesting the applicability of this LTD-blocking peptide

to various brain regions. Another LTD-interfering peptide, NR2B9c, is derived from the intracellular C-terminal of NR2B, and was recently found to be able to block the first step of the induction of hippocampal NMDAR-dependent LTD (Wang et al, preliminary observation). In our present study, both LTD-blocking peptides were fused to the cell-membrane transduction domain of the HIV Tat sequence (Tyr-Gly-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg), which enables the peptides to pass through the blood brain barrier and cell membrane in the central nervous system after systemic injection. A few studies have shown that Tat-fused peptides, after i.v. or i.p. infusion, could be either detected or effective and functioning in some brain regions including cortex, hippocampus and striatum (Aarts et al., 2002; Brebner et al., 2005; Ji et al., 2006). However, neither peptide exhibited the capacity to significantly alter innate anxiety in rat NSF task, suggesting that the development of innate anxiety does not require LTD production. Taken together, our study using both NR2B antagonists and LTD-blocking peptides suggests that NR2B-containing NMDARs participate in the generation of innate anxiety through intracellular mechanism(s) that does not involve the induction of LTD.

In summary, these results provide further evidence that NR2B-containing NMDARs are critically involved in the process of innate anxiety disorders, and the NR2B-containing NMDARs in the amygdala also participate in the innate anxiety expression as well. The anxiolytic effect of NR2A-preferring antagonist NVP-AAM077 was not observed in this study. The precise mechanism(s) underlying these effects need to be investigated further.

5. FUTURE RESEARCH

To make this project a complete and convincing story, the following work will be completed in the near future.

Firstly, positive controls of NVP-AAM077, Tat-GluR2-3Y and Tat-NR2B9c will be conducted. Fox et al. (2007) showed that after i.p. infusion NVP-AAM077 could be effective in the hippocampus, but the effectiveness of this chemical in the amygdala need to be further proved. We will perform *in vivo* electrophysiological recording to see if the i.p. administration of NVP-AAM077 will block the induction of amygdaloid LTD. The similar experiment will be conducted with systemic injection of Tat-GluR2-3Y or Tat-NR2B9c to prove that after i.p. infusion these Tat-fused peptides could enter the amygdala and be functioning in the amygdala.

Then more experiments will be performed to further explore the different intracellular pathways underlying the distinct effects of systemic vs. intra-BLA injection of NR2B antagonist Ro25-6981. A study (Cratty and Birkle, 1999) showed that glutamate and NMDA stimulated corticotrophin-releasing factor (CRF) release in cultured rat amygdala neurons, and this effect could be blocked by the NMDAR antagonist AP-5 or MgCL₂. These results implicated that the inhibition of CRF release in the amygdala may be a possible mechanism for the anxiolytic effects of intra-BLA injection of NMDAR antagonist Ro25-6981 observed in this study. Since the anxiogenic effect of i.p. injection of NR2B antagonists is not through the LTD induction pathway, we will have to explore other possible mechanism(s). We noticed that Tohda et al. (2009) reported that the phosphoinositide-3 kinase (PI3K) deficit mice showed increased anxiety in the EPM test. Thus the anxiogenic effect of systemic

administration of NR2B antagonists may be via inhibition of intracellular PI3K pathway.

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