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Group II Metabolic Glutamate Receptors in the Basal Amygdala Regulate Sleep and Fear-Induced Alterations in Sleep

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ABSTRACT

GROUP II METABOTROPIC GLUTAMATE RECEPTORS
IN THE BASAL AMYGDALA REGULATE SLEEP AND
FEAR-INDUCED ALTERATIONS IN SLEEP

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Eastern Virginia Medical School and Old Dominion University, 2010
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Glutamate is a major excitatory neurotransmitter in the brain and it has been recognized as playing an essential role in activating and maintaining arousal. Group II metabotropic glutamate (mGlu II) receptors are expressed in the amygdala, a brain structure important in the regulation of stress and anxiety as well as in the regulation of sleep and arousal. Our lab has found that the central nucleus of the amygdala (CNA) is involved in the emotional modulation of sleep. The basal amygdala (BA), which has direct connections with the CNA, is involved in conditioned fear and fear extinction. However, the potential role of the BA in the regulation of sleep has received little attention. In this study, we investigated the role of mGlu II receptors in sleep and stress-induced alterations in sleep.

In work detailed in chapter II, either the mGlu II receptor agonist LY379268 (LY37) or antagonist LY341495 (LY34) was microinjected into the BA in rats. The effects on sleep were recorded and analyzed. It was found that LY37 suppressed rapid eye movement sleep (REM) without significantly altering non-REM sleep (NREM) or total sleep. In contrast, microinjection of LY34 at a high concentration (60 nM) increased arousal by suppressing REM and NREM.

In work detailed in chapter III, rats received conditioned fear and extinction training and LY34 or vehicle was injected into the BA immediately before fear extinction training. It was found that rats injected with LY34 demonstrated greater fear responses in the earlier periods of extinction training and showed less fear responses later in the session when compared to the control group. LY34 also ameliorated sleep disturbances following fear extinction training.

These results demonstrate that the BA may be a brain area that exerts a regulatory effect on sleep, especially REM. mGlu II receptors in the BA may be essential for regulating sleep. Suppression of these receptors may increase arousal and contribute to the improved performance of fear extinction. These data expand our knowledge on the role of the amygdala in the regulation of sleep. They may also provide prospects for the treatment of anxiety disorders, such as posttraumatic stress disorder (PTSD).

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CHAPTER I

INTRODUCTION

Introduction to the Study

Stress alters homeostasis and induces multiple physiological responses. It is considered to be an important contributor to various health problems (Korte et al., 2005). The effects of stress on behaviors during wakefulness can be obvious. However, sleep in the period following a stressful event may also be affected. In recent years, the effects of stress on sleep have received considerable attention and a number of studies have been performed. In our lab, conditioned fear has been used as a model for studying stress related to fear and anxiety and its impact on sleep (Sanford et al., 2003; Tang et al., 2005). We focus on the amygdala because it is an important emotional center in the brain (LeDoux, 1992) and it plays an important role in conditioned fear (Davis, 1997). In conditioned fear, both training with footshock and shock associated contexts can alter subsequent sleep patterns, especially rapid eye movement sleep (REM) (Sanford et al., 2003). The amygdala, in particular the central nucleus of the amygdala (CNA), plays a significant role in regulating sleep and in mediating the effects of stress on sleep (Sanford et al., 1998; Sanford et al., 2002; Tang et al., 2005). In addition, the basal amygdala (BA) has a direct connection with the CNA (Davis and Whalen, 2001). Blockage of the BA impairs fear learning (Ono et al., 1995; Maren et al., 1996), suggesting that the BA is

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involved in the regulation of emotion. Furthermore, electrical lesion of the basolateral amygdala (BLA, a complex that includes the BA, the lateral amygdala (LA) and the accessory basal amygdala (AB)) suppresses wakefulness and increases non-REM sleep (NREM) and REM, suggesting that the BA may be important in the amygdalar regulation of sleep.

Glutamate is the major neurotransmitter in the amygdala, especially in the BA (Sah et al., 2003). It is implicated in stress and anxiety (Bergink et al., 2004; Cortese and Phan, 2005). For example, suppression of N-methyl-D-aspartate (NMDA) receptors in the BLA impairs the acquisition and expression of contextual fear (Maren and Fanselow, 1995). In recent years, pharmacological research has revealed that metabotropic glutamate (mGlu) receptors may also play a role in the regulation of stress and anxiety (Palucha and Pilc, 2007). Group II metabotropic glutamate (mGlu II) receptors, which are expressed in the BA (Ohishi et al., 1993; Ohishi et al., 1993), have been proposed to serve as autoreceptors that suppress glutamate release when activated and they have been implicated in the modulation of synaptic transmission (Wright et al., 2001). It has also been found that systemic injection of mGlu II receptor agonists or antagonists can alter sleep (Feinberg et al., 2002; Feinberg et al., 2005). Therefore, in this study, we manipulated mGlu II receptors in the BA in order to investigate their potential role in regulating sleep and in mediating the effects of stress on sleep.

In the following sections, we provide background information for major components of our studies. First, we introduce glutamate as a major excitatory neurotransmitter in the brain and how it may function through specific glutamate receptors including ionotropic (iGlu) and mGlu receptors. Specifically, we focus on mGlu II receptors that are expressed

in the amygdala, an important brain area involved in the regulation of emotion. We also provide background information for fear conditioning and fear extinction.

1. Glutamate is an Excitatory Neurotransmitter

Glutamate is the major excitatory neurotransmitter in the brain, and it is reported to be released in over half of all brain synapses (Monaghan et al., 1989). Due to the existence of the blood-brain barrier (endothelial cells of blood vessels in the brain which restrict the diffusion of large molecules from blood into the brain) glutamate is poorly transported from blood to cerebrospinal fluid. Therefore, brain glutamate must be synthesized from neurons in local areas (Cooper, 2003). In fact, in nerve terminals, glutamate is synthesized from two major sources: from glucose via the Krebs cycle and transamination of α-oxoglutarate (Fig. 1); or from glutamine transported by glial cells and locally metabolized by the mitochondrial enzyme, glutaminase, into glutamate (Fig. 2).

In nerve terminals, glutamate is packaged into synaptic vesicles. Upon the depolarization of a nerve terminal, synaptic vesicles containing glutamate move to the synaptic membrane and glutamate is released through calcium dependent exocytosis. Reuptake of released glutamate from the synaptic cleft is accomplished by neuronal ($GT_{[n]}$) and glial ($GT_{[g]}$) Na^+-coupled glutamate transporters. Glutamate taken by glial cells is converted into glutamine, which is transported into the cerebrospinal fluid and serves as a precursor for glutamate synthesis (Fig. 2).

2. Glutamate is Implicated in Stress and Anxiety

As a major excitatory neurotransmitter in the brain, glutamate is thought to play an

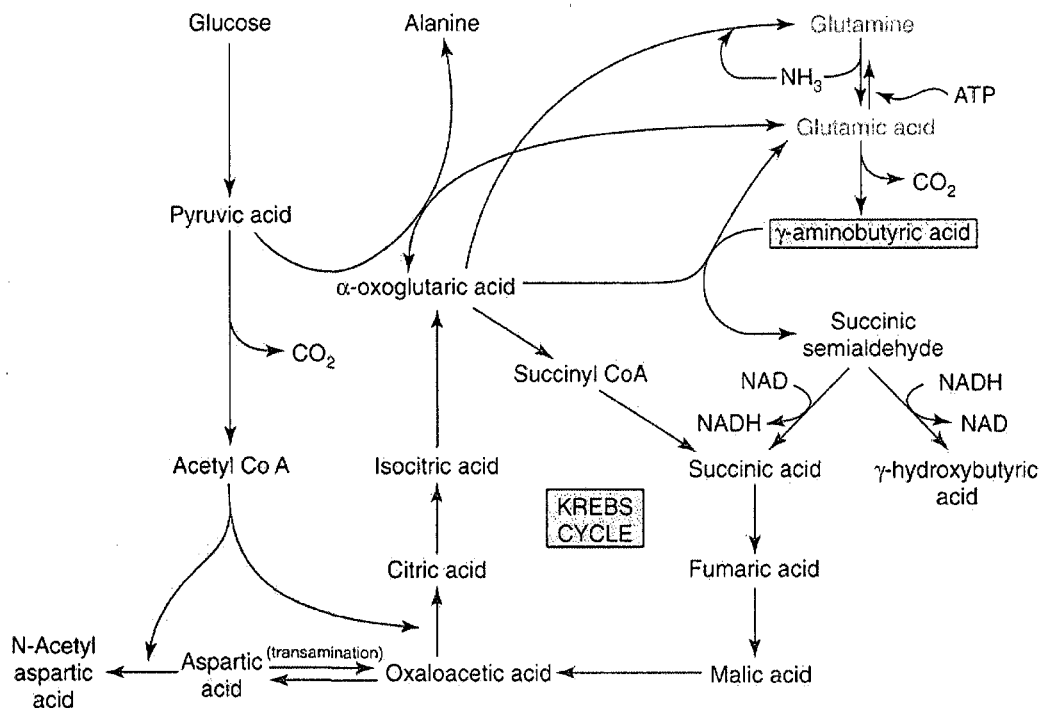


Fig. 1 - Pathway of glutamate synthesis from glucose via the Krebs cycle and transamination of α-oxoglutarate [From Cooper, 2003].

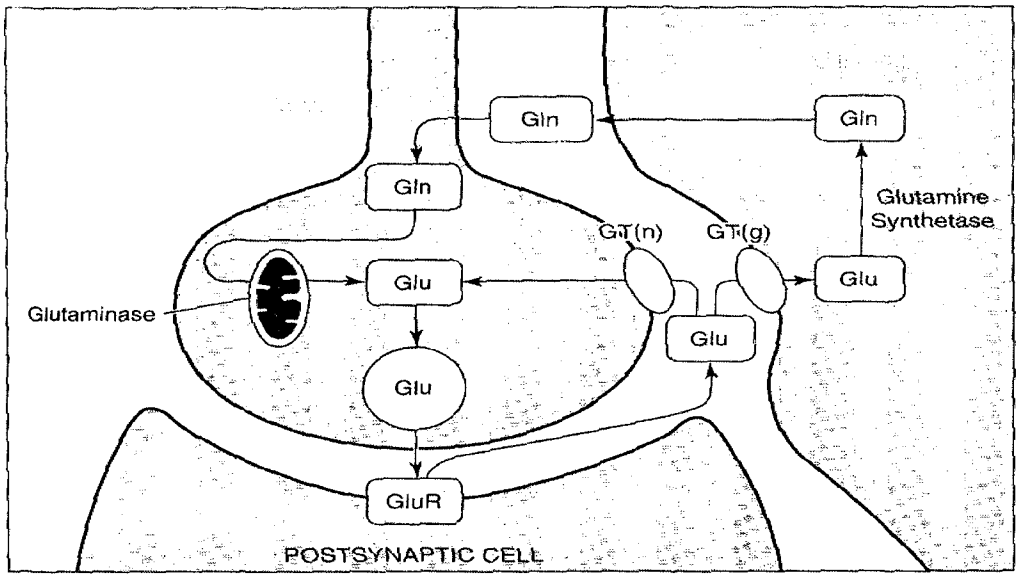


Fig. 2 - Pathway of glutamate synthesis and utilization. Released glutamate in the synaptic cleft is removed by neuronal ($GT_{[n]}$) and glial ($GT_{[g]}$) Na^+-coupled glutamate transporters. In glial cells, glutamine is synthesized from glutamate by the enzyme glutamine synthetase and released into the cerebrospinal fluid. Neuronal terminals can take up glutamine and convert it into glutamate through hydrolysis by the mitochondrial glutaminase [From Cooper, 2003].

important role in the development of stress and anxiety. Studies from various labs have reported that chronic immobilization stress increases glutamate release and uptake in the hippocampus and the prefrontal cortex (PFC) (Gilad et al., 1990; Lowy et al., 1995; Fontella et al., 2004). In addition, chronic immobilization stress can also increase the messenger RNA expression level of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors in the hippocampus (Schwendt and Jezova, 2000). Furthermore, injection of the NMDA receptor antagonist, D,L-2-amino-5-phosphonovalerate (APV), into the BLA impairs both acquisition and expression of conditioned fear in rats (Maren et al., 1996).

3. Glutamate is Involved in Maintaining Arousal

Glutamate is involved in activating and maintaining arousal. Glutamate is found in high concentration in brain areas involved in activating and maintaining arousal, such as the reticular activating system (Jones, 2005). The release of glutamate in cerebral cortex reaches its high peak during the cortical activation of spontaneous wakefulness or activation induced by the stimulation of the midbrain reticular formation (Jasper et al., 1965). Furthermore, microdialysis studies have found enhanced glutamate release in the rostromedial medulla during REM (Kodama et al., 1998). In addition, glutamate antagonists such as ketamine have sedative effects (Mayer and Westbrook, 1987).

4. Overview of Glutamate Receptors

Glutamate acts through stimulation of two groups of receptors: iGlu and mGlu receptors. Three types of iGlu receptors have been discovered that are named based on their respective agonists: AMPA receptors, NMDA receptors and kainate receptors. These

receptors are nonselective ion channels which open and allow the passage of Na^+, K^+ and Ca^{2+} when the receptors are activated by binding with glutamate. While iGlu receptors provide fast and massive responses for signal transduction, mGlu receptors induce slower responses by generating signal cascades through G proteins. mGlu receptors are a family of eight G-protein coupled receptors: mGlu1 to mGlu8 receptors. They are classified into three groups according to their homology and pharmacology: Group I mGlu (mGlu I) receptors (mGlu1 and mGlu5), mGlu II receptors (mGlu2 and mGlu3) and Group III mGlu (mGlu III) receptors (mGlu4, mGlu6, mGlu7 and mGlu8). mGlu I receptors are positively coupled to phospholipase C whereas mGlu II receptors and mGlu III receptors are negatively coupled to adenyl cyclases. mGlu receptors provide a mechanism that modulates the activity of glutamate at the same synapses with fewer side effects (Conn and Pin, 1997). Therefore, ligands of mGlu receptors can be used to manipulate glutamate release at the synaptic level and to investigate the role of glutamate in neuronal plasticity.

All mGlu receptors are homodimers that need glutamate for their functions (Kunishima et al., 2000). They have similar structures that can be illustrated by the detailed structure of the mGlu1 receptor (Fig. 3). The mGlu1 receptor has a large extracellular domain which is composed of a ligand-binding region (LBR) and a cysteine-rich region (CR) that combines LBR with the transmembrane region (TM). The LBRs of the mGluR1 homodimer are composed of two subdomains: LB1 and LB2. The LBRs of the homodimer can take either of two conformations: open or closed. Binding of glutamate can stabilize the closed conformation, which triggers LB2 subdomains to combine together and thus activate the receptor.

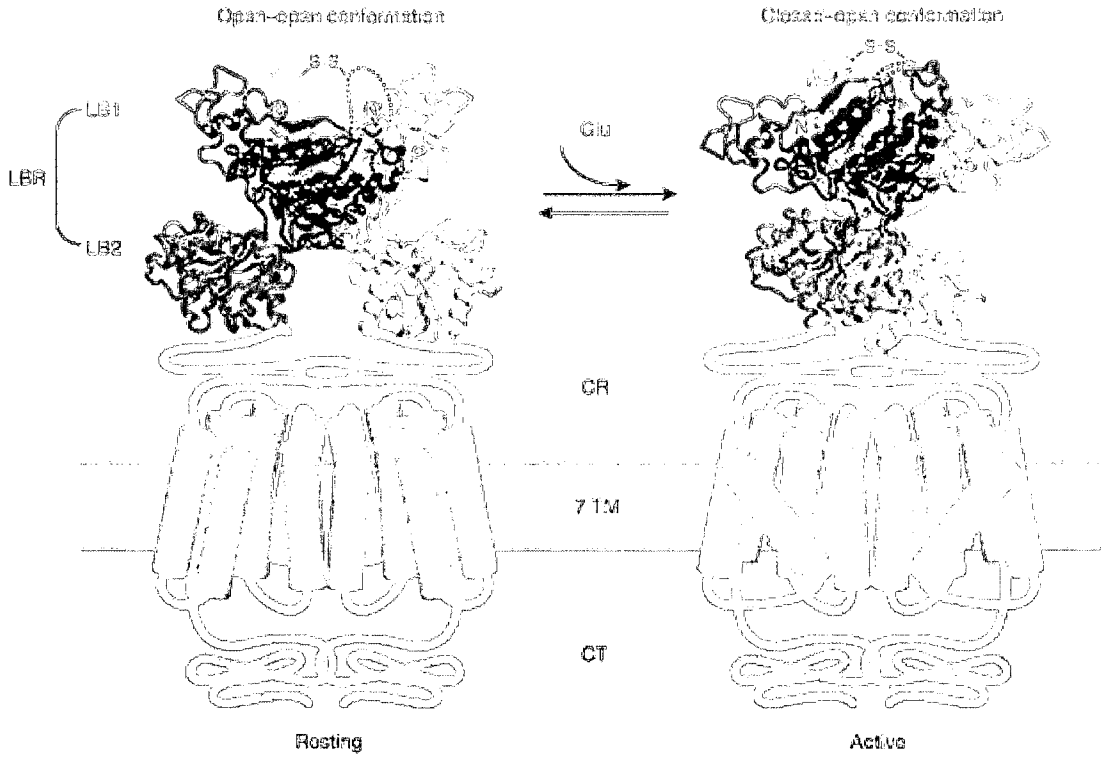


Fig. 3 - Structure of the mGlu1 receptor. The receptor has a large extracellular domain which is composed of a ligand-binding region (LBR) and a cysteine-rich region (CR) that combines LBR with the transmembrane region (7 TM). The LBRs of the mGlu1 homodimer are composed of the two subdomains LB1 and LB2. The LBRs can take either one of two conformations: open or closed. Binding of glutamate can stabilize the closed conformation, which triggers LB2 subdomains of the homodimer to combine together and activate the receptor. CT, carboxyl terminus; Glu, Glutamate [From Moepps and Fagni, 2003].

As mGlu receptors may modulate glutamate release at the synaptic level, the location of each type of receptor may be an important issue for the role of each receptor type (Ottersen and Landsend, 1997). Immunostaining has revealed that mGlu I receptors (mGlu1 and mGlu5) are primarily located postsynaptically (Lujan et al., 1997) whereas mGlu III receptors (mGlu4, mGlu7, and mGlu8) are mainly located presynaptically (Shigemoto et al., 1997). mGlu II receptors (mGlu2 and mGlu3) are located presynaptically and postsnaptically in the synaptic cleft (Petralia et al., 1996). Electron microscopy has revealed that mGlu2 receptors are located primarily in presynaptic membranes of nerve terminals. Furthermore, they are located far away from the active zones (Shigemoto et al., 1997). mGlu3 receptors are also expressed in glial cells and may contribute to the uptake of glutamate (Mineff and Valtschanoff, 1999) (Fig. 4).

Of these glutamate receptors, we focus on mGlu receptors, which provide slower but long-lasting modulatory effects on synaptic transmission that may possibly be the underlying mechanism for the development of anxiety. Specifically, we are interested in mGlu II receptors, which are positioned at the area far away from active zone and may only act during stressful situations that trigger released glutamate to a sufficient level to diffuse to and activate these receptors.

5. mGlu II Receptors

In recent years, much attention has been paid to mGlu II receptors, which are considered to be a potential drug target for the treatment of anxiety disorders (Swanson et al., 2005). mGlu II (mGlu2 and mGlu3) receptors are expressed in brain structures implicated in emotion, such as the amygdala, the hippocampus and the PFC (Tamaru et al., 2001; Wright et al., 2001). Immunological staining has revealed that mGlu II

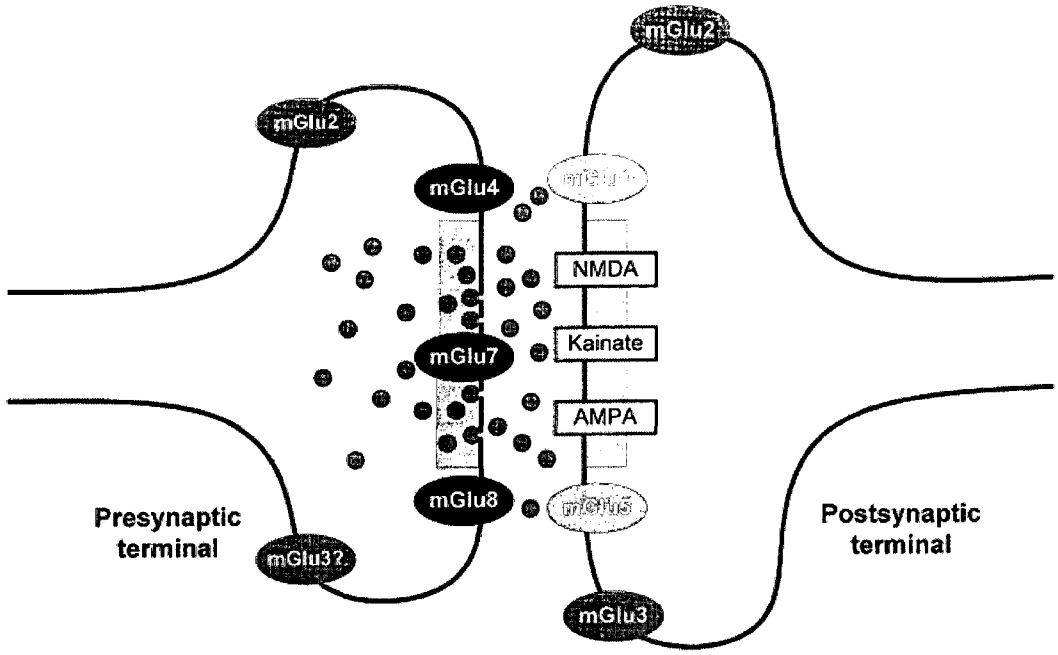
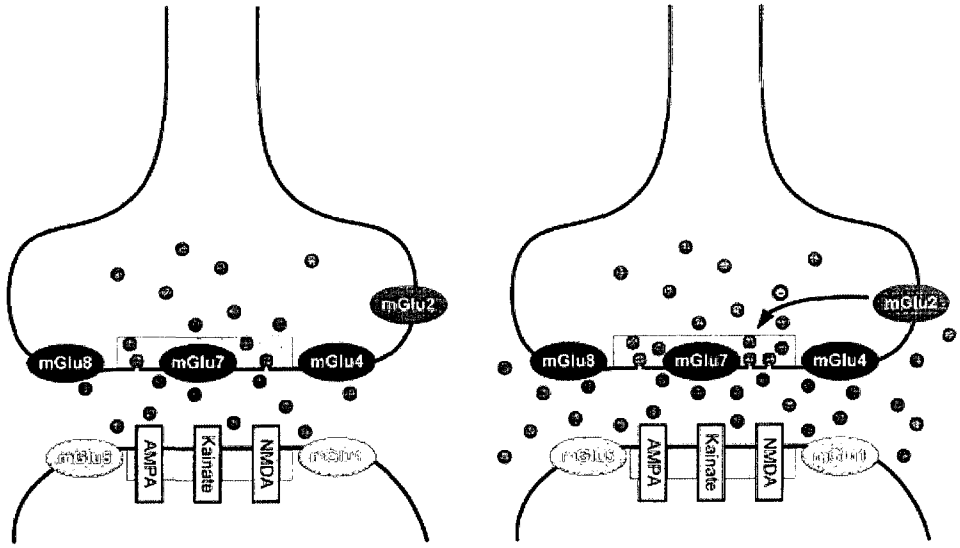


Fig. 4 - Localization of mGlu receptors at a theoretical synapse in the central nervous system. mGlu I receptors (mGlu1 and mGlu5) are primarily located postsynaptically in synapses whereas mGlu III receptors (mGlu4, mGlu7, and mGlu8) are mainly located presynaptically. mGlu II receptors (mGlu2 and mGlu3) are located presynaptically and postsynaptically in the synaptic cleft. Furthermore, they are located far away from the active zones. There are only mGlu7 receptors located in the active zones of the presynaptic terminal [From Cartmell and Schoepp, 2000].

receptors are mainly located in presynaptic terminals (Shigemoto et al., 1997). mGlu II receptors are negatively coupled with adenylyl cyclases and the activation of mGlu II receptors will inhibit forskolin-stimulated cyclic AMP formation (Conn and Pin, 1997). Although the function of mGlu3 receptors is still under investigation, it is proposed that mGlu2 receptors serve as glutamate autoreceptors and are responsible for decreasing glutamate release level (Fig. 5). Under conditions of high glutamate release, mGlu2 receptors will be activated and thus generate an inhibitory effect on glutamate release (Yokoi et al., 1996; Scanziani et al., 1997). The reduction of glutamate release may provide a mechanism for the anxiolytic-like effects of mGlu II receptor agonists.

6. mGlu II Receptor Agonists: LY354740 (LY35) and LY379268 (LY37)

mGlu II receptor agonists include LY35 and LY37, which show low nanomolar affinity for both receptor subtypes and are systemically active (Palucha and Pilc, 2007). LY35 has been shown to have significant anxiolytic-like potentials in several models of anxiety. For example, oral administration of LY35 induces a dose-dependent reduction of forced swimming in rats (Helton et al., 1998). LY35 also increases open-arm activity in the elevated plus maze in mice (Monn et al., 1997). Intraperitoneal injection of LY35 significantly reduces lactate-induced panic-like responses in panic-prone rats (Shekhar and Keim, 2000). The anxiolytic effect of LY35 has also been confirmed in humans. Administration of LY35 decreases fear-potentiated startle in healthy volunteers (Grillon et al., 2003). The mechanism underlying the anxiolytic effect of LY35 may rely on its stimulation of presynaptic mGlu2/3 receptors that inhibit glutamate release (Imre, 2007). LY37 is a derivative of LY35, and is considered to be an even more potent and selective agonist than LY35. *In vivo* microdialysis has been used to assess the ability of LY37 (10



Low stimulation **High stimulation**

Fig. 5 - Proposed theory that mGlu2 receptors serve as autoreceptors of glutamate release. Under normal conditions, glutamate does not reach a level sufficient for diffusing from the release site to activate mGlu2 receptors located far away from the active zone. However, under stress, released glutamate may reach such a high level that it can diffuse to perisynaptic sites and activate mGlu2 receptors and hence induce inhibition of glutamate release [From Cartmell and Schoepp, 2000].

mg/kg) to reach receptor active level in the PFC in rats. It was found that LY37 reaches levels of 600-800 nM in the extracellular fluid of the brain within 1 hour, and then declines over the next 5 hours to a level less than 30 nM (Imre, 2007). These data suggest that, with systemic administration, LY37 can reach brain structures such as the PFC and can activate mGlu II receptors for more than 6 hours. Compared with LY35, fewer studies have evaluated the effects of LY37 on anxiety and stress. However, evidence suggests that LY37 has anxiolytic effects. Systemic administration of LY37 attenuates the increase of norepinephrine in the PFC in rats exposed to an elevated platform (Lorrain et al., 2005). LY37 also impacts sleep: systemic administration of LY37 in rats generates a dose-dependent suppression of both REM and fast EEG activity during NREM (Feinberg et al., 2002).

7. mGlu II Receptor Antagonist: LY341495 (LY34)

LY34 is a nanomolar potent antagonist of mGlu II receptors and can reverse agonist-induced inhibition of forskolin-stimulated cAMP formation at mGlu II receptors (Kingston et al., 1998). mGlu II receptors are negatively coupled to inhibitory G protein (G_i) and activation of these receptors can inhibit the production of cAMP. These data suggest that LY34 can block the function of mGlu II receptors as revealed by cAMP production. For example, low doses (0.3 mg/kg, ip) of LY34 can block the anxiolytic effects of the agonist LY35 in the elevated plus maze (O'Neill et al., 2003). These data confirm that LY34 is a specific mGlu II antagonist. It has been reported that systemic administration of LY34 enhances mouse anxiety during the elevated plus maze test (Linden et al., 2005). Furthermore, LY34 induces enhancement of widespread c-Fos expression in the brain (Linden et al., 2005). LY34 can also alter sleep. Administration of

LY34 increases arousal and suppresses REM and NREM in rats (Feinberg et al., 2005).

8. The Amygdala

The amygdala is an almond-like mass of gray matter located in the medial temporal lobes of various mammals, including human and rodents. The amygdala is structurally composed of multiple, distinct nuclei which have enriched internuclei and intranuclei connections. These nuclei are classified into three major groups: (a) the BLA group, composed of the LA, the BA and the AB, has extensive connections with the cerebral cortex including the orbital and the medial prefrontal cortex (mPFC); (b) the medial group has connections with the olfactory bulb and the olfactory cortex; (c) the central and anterior group has connections with the brainstem, the hypothalamus and visceral sensory structures (Fig. 6). Therefore, the amygdala connects cortical regions that are responsible for sensory information processing with hypothalamic and brainstem regions that are involved in the regulation of emotional behavior and sleep (Sah et al., 2003).

9. The Amygdala Is Implicated in the Regulation of Stress and Sleep

The amygdala has a critical role in conditioned fear and anxiety. In humans stimulation of the amygdala generates anxiety-like responses (Feindel and Penfield, 1954), while lesions of the amygdala attenuate anxiety responses (Narabayashi et al., 1963). Animal studies also indicate a primary role of the amygdala in fear and anxiety (Davis, 2000). The BA is particularly associated with fear and anxiety. Chemical and electrical stimulations of the BA generate anxiety-like responses (Gelsema et al., 1987; al Maskati and Zbrozyna, 1989). Moreover, the BA contains a high concentration of benzodiazepine receptors (File, 2000). Microinjection of benzodiazepine derivative,

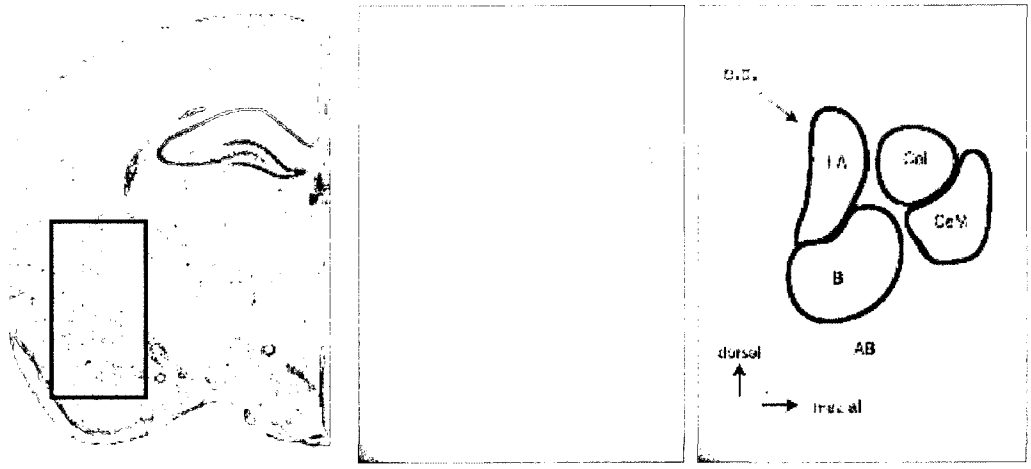


Fig. 6 - An illustration of the amygdala region in coronal sections. Left panel: a Nissl-stained hemisection of a rat brain. Middle panel: the region containing the basolateral complex (BLA) and the central nucleus (CNA). Right panel: nuclei of the lateral (LA), the basal (B), the accessory basal (AB) nucleus and the CNA. CeL: the lateral nuclei of the CNA; CeM: the medial nuclei of the CNA [From Sah et al., 2003].

midazolam, into the BA elicits anxiolytic effects in rats in the water lick conflict test (Scheel-Kruger and Petersen, 1982). These data suggest that the BA may play an important role in regulating anxiety responses.

The amygdala is also implicated in the regulation of sleep. The amygdala receives various sensory inputs into the LA and the BA. These nuclei in turn send efferent projections to the CNA, which projects to structures involved in the regulation of sleep, such as the brainstem, the basal forebrain and the preoptic nucleus of the hypothalamus. Evidence shows that the amygdala can modulate sleep, especially REM. For example, electrical stimulation of the amygdala increases REM (Smith and Miskiman, 1975). However, most of this work focuses on pharmacological manipulations targeting the CNA. For example, microinjection of the cholinergic agonist, carbachol, into the CNA significantly decreases total REM and electroencephalogram (EEG) power in the 5.5-10 Hz band during REM without significantly altering NREM in rats (Sanford et al., 2006). Microinjection of prolactin into the CNA decreases NREM (Sanford et al., 1998). Microinjections of muscimol, a $GABA_A$ agonist, into the CNA induces selective decreases in total REM and number of REM episodes without significant changes in NREM or wakefulness whereas microinjections of bicuculline, a $GABA_A$ antagonist, into the CNA produces significant increases in REM (Sanford et al., 2002). These data suggest that the amygdala has an important role in the regulation of sleep.

In contrast to the inhibitory role of GABAergic system, glutamatergic neurons constitute the major excitatory system in the amygdala. In this study, we investigated the possible role of mGlu II receptors in the BA for the regulation of stress and sleep.

10. Fear Conditioning

Fear conditioning is a powerful classical paradigm that is used for study of fear and anxiety. During training in contextual fear conditioning, animals make associations between an aversive, fear-inducing stimulus, usually footshock, and the environment in which it is received. Afterwards, the animals exhibit fear responses when they experience the context alone. This is defined as contextual fear. Both the hippocampus and the BA are essential, as damage to these areas impairs contextual fear (Maren and Fanselow, 1995; Maren et al., 1997). The hippocampus has projections to the BA and it is proposed that information regarding environmental context may be processed in a circuit from the hippocampus to the BA and the AB where an association may be formed between conditioned (CS) and unconditioned (US) stimuli (LeDoux, 2000). The BA and the AB in turn project to the CNA, which projects to the brainstem areas that control the expression of fear responses (Davis and Whalen, 2001). The CNA has direct projections to the nucleus of the solitary tract (NTS) and the dorsal motor nucleus of the vagus (DMN), which influence cardiovascular responses. The CNA also sends projections to the periaqueductal gray area which regulates freezing responses (defined as the absence of body movement except for respiration), an index of fear memory.

Many studies have explored the role of the CNA in fear conditioning using techniques including electrical or chemical lesion, electrical stimulation and local infusion of various drugs. However, these methods may influence projections that go through the CNA. As shown in Fig. 7, biotinylated dextran amine (BDA), an anterograde tracer, was infused into the BA and the brain was sectioned to study labeled terminals. Many fibers were stained in the CNA, while many other fibers were found to

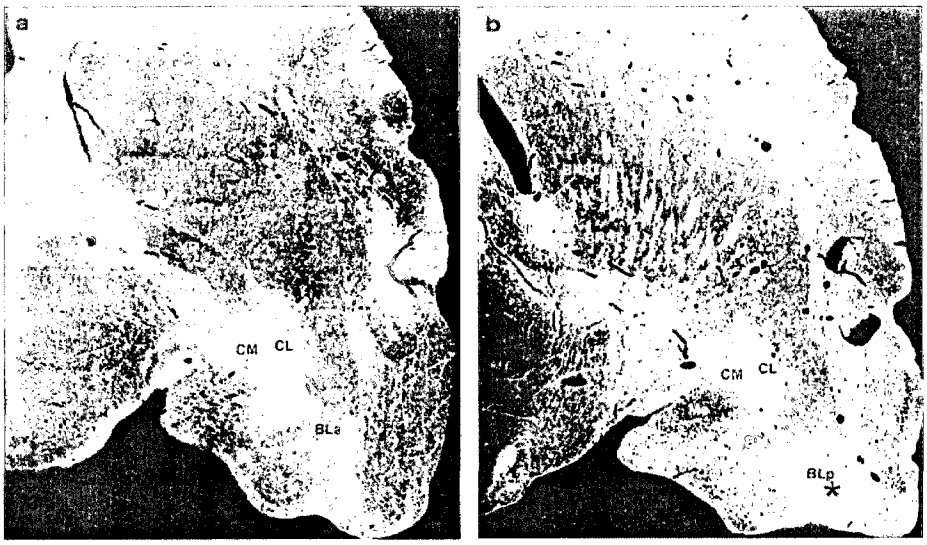


Fig. 7 - Projections of the basolateral nucleus (BL) of the amygdala revealed by biotinylated dextran amine (BDA) staining. CM: the medial nuclei of the central nucleus of the amygdala (CNA); CL: the lateral nuclei of the CNA; BLa: the anterior part of the BL; BLp: the posterior part of the BL; BNSTal: the anterior part of the bed nucleus of the stria terminalis (BNST); BNSTpl: the posterior part of the BNST [From Davis and Whalen, 2001].

pass through the CNA from the BA and to reach the bed nucleus of the stria terminalis (BNST). Therefore, electrical activation or lesions of the CNA may affect projections from the BA. In addition, the BNST also has many projections to the hypothalamus and the brain stem; thus, the BA may have the ability to activate the same targets through the BNST as it does through the CNA (Davis and Whalen, 2001).

Therefore, it is likely that the BA has an impact on the regulation of fear responses. Since the major neurons in the BA are pyramidal cells that use glutamate as a neurotransmitter (Sah et al., 2003), we expect glutamate in the BA should play a role in fear conditioning. As mGlu II receptors are expressed in the BA, we used their antagonist, LY34, to manipulate mGlu II receptor function to determine if mGlu II receptors play a role in fear conditioning.

11. Fear Extinction

After conditioned fear is established, repeatedly presenting the shock context without aversive consequences will typically result in the context losing the ability to induce fear responses. This process is defined as fear extinction (Quirk and Mueller, 2008) (Fig. 8). Research has explored the neuronal mechanism underlying the acquisition and consolidation of fear memory. It has been proposed that acquisition and storage of information may rely on circuits which transmit both CS (such as the context) and US (such as footshock) information to the BA. Expression of conditioned fear depends on the pathway that transmits information regarding the CS to the BA and the CNA (Davis and Whalen, 2001). Interestingly, fear extinction is considered as new learning that the CS is no longer associated with the US (Bouton, 2002). The interaction between the mPFC and

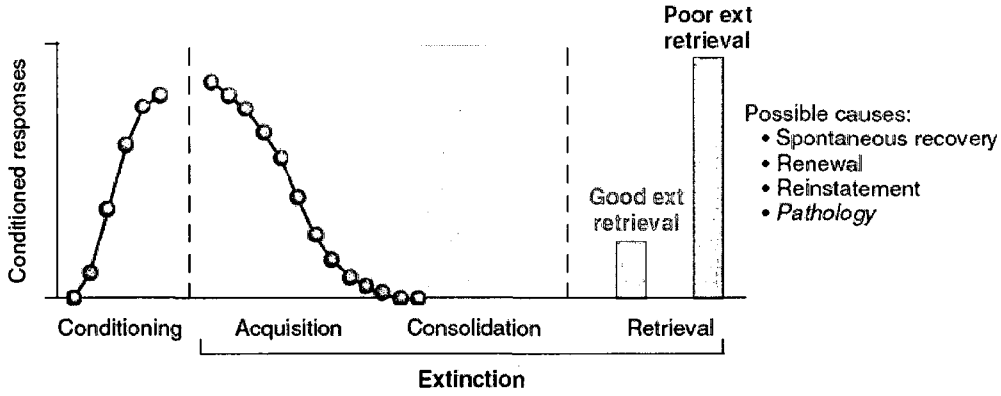


Fig. 8 - Illustration of fear extinction learning. During fear extinction, acquisition means a decrease in conditioned responses to the presentation of the conditioned stimulus. Consolidation is a process during which a long-term extinction memory is formed. Retrieval of extinction happens when the subject encounters the conditioned stimulus at a later time [From Quirk and Mueller, 2008].

the amygdala may be involved in fear extinction (Quirk et al., 2000). Lesion studies have revealed that the mPFC is essential for the retrieval of extinction. The infralimbic neurons of mPFC (IL) fire only when rats retrieved extinction on the day following extinction training (Milad and Quirk, 2002). The IL sends extensive excitatory projections into the LA and the intercalated cell masses (ITC), while the prelimbic neurons of mPFC (PL) send heavy stimulatory inputs into the BA (McDonald et al., 1996). The BA has been found to send back projections to the PL, which projects to the IL (Shinonaga et al., 1994). Interestingly, stimulation of the IL induced neuronal activity in the LA whereas stimulation of the PL induced neuronal activity in the BA (Rosenkranz and Grace, 2001). Considering that projections from the mPFC to the amygdala are mainly excitatory (Smith et al., 2000), the inhibitory effects of the mPFC on the amygdala may be attributed to the enrollment of inhibitory interneurons in the amygdala (Rosenkranz and Grace, 2001; Quirk and Gehlert, 2003). These data suggest that the BA may be an essential site for fear extinction.

In summary, stressors and contextual reminders of stressors can generate significant changes in behavior and sleep. The amygdala plays an important role in the regulation of fear and anxiety. It has also been found to play a role in regulating sleep. Activation of the CNA has been found to facilitate REM generation and the inactivation of the CNA impedes REM generation. However, the role of the BA in regulating sleep has received less attention.

Moreover, the BA is also involved in the regulation of fear extinction learning. In addition, mGlu II receptors are expressed in the BA and may provide a pathway for manipulation of glutamate release. However, the role of mGlu II receptors in regulating

stress-induced alterations in sleep has not been explored. In this study, we examined the effects of LY37 and LY34 infusions into the BA on sleep architecture and amounts. We also evaluated the effects of LY34 on contextual fear conditioning and fear extinction and determined the effects of infusion of LY34 into the BA on stress-induced alterations in sleep.

CHAPTER II

GROUP II METABOTROPIC GLUTAMATE RECEPTORS IN THE BASAL AMYGDALA REGULATE SLEEP

1. Introduction

Glutamate is the major excitatory neurotransmitter in the brain and it plays an essential role in maintaining wakefulness (Jones, 2005). It is found in high concentration in neurons in the brainstem reticular activating system (Jones, 2005). In addition, the release of glutamate in the cerebral cortex reaches its highest peak during the cortical activation in spontaneous wakefulness or arousal produced by the stimulation of the midbrain reticular formation (Jasper et al., 1965).

Glutamate acts through two major groups of receptors: ionotropic glutamate (iGlu) receptors and metabotropic glutamate (mGlu) receptors. The stimulation of iGlu receptors opens an ionic pathway across the postsynaptic membrane and produces a rapid synaptic response. In contrast, mGlu receptors stimulate cyclic AMP production and induce metabolic changes which are relatively slow (Cartmell and Schoepp, 2000). Eight types of mGlu receptors have been cloned, which are classified into three groups (I, II, III) according to their sequence homology, second messenger mechanism and pharmacologic activity (Palucha and Pilc, 2007). mGlu II receptors are negatively coupled with adenylyl cyclases and are located mainly outside the active zone area of presynaptic terminals. They have been implicated in the modulation of synaptic transmission and it has been proposed that they serve as autoreceptors that suppress glutamate release when activated (Wright et al., 2001). mGlu II receptors may also affect the release of other

neurotransmitters, such as dopamine, γ-Aminobutyric acid (GABA) and serotonin (Cartmell and Schoepp, 2000).

Work at the systemic level has implicated mGlu II receptors in the regulation of sleep. Subcutaneous injection of the mGlu II receptor agonist, LY379268 (LY37), induces a dose-dependent suppression of rapid eye movement sleep (REM) following administration (Feinberg et al., 2002). In addition, subcutaneous injection of the mGlu II agonist, LY354740 (LY35), dose-dependently suppresses REM and prolongs its onset latency in both rats and mice, but does not alter REM in mGlu2 receptor negative mice (Ahnaou et al., 2009). Moreover, systemic administration of the mGlu II receptor antagonist, LY341495 (LY34), increases arousal by suppressing REM and non-REM sleep (NREM) (Feinberg et al., 2005). However, the potential neural site(s) that mediates these changes in sleep has not been determined.

mGlu II (mGlu2 and mGlu3) receptors, are expressed in the amygdala (Ohishi et al., 1993; Ohishi et al., 1993), a limbic structure increasingly implicated in the regulation of arousal and sleep (Sanford et al., 1998; Deboer et al., 1999; Morrison et al., 2000; Sanford et al., 2002; Jha et al., 2005; Tang et al., 2005) as well as having a demonstrated role in emotion (Davis and Whalen, 2001). Early work demonstrates that electrical stimulation of the amygdala increases REM (Smith and Miskiman, 1975). The central nucleus of the amygdala (CNA) projects to brainstem structures involved in REM regulation and generation (Krettek and Price, 1978; Takeuchi et al., 1982; Petrov et al., 1994) and most recent research has focused on its role in regulating sleep (Sanford et al., 2002; Tang et al., 2005; Sanford et al., 2006). However, involvement of other regions of the amygdala in the regulation of sleep is suggested by reports that bilateral electrolytic

and chemical lesions of the basolateral amygdala (BLA, a complex that includes the basal amygdala (BA), the lateral amygdala (LA) and the accessory basal amygdala (AB)) increase total REM and NREM amounts in rats (Zhu et al., 1998) and that bilateral chemical lesions of the amygdala produce more consolidated sleep in chair-restrained Rhesus monkeys (Benca et al., 1992). Electrical and chemical stimulation of the BLA also increases low voltage, high frequency activity in the cortical EEG and decreases NREM and total sleep time, respectively (Dringenberg and Vanderwolf, 1996; Zhu et al., 1998).

The BA contains mGlu II receptors (Wada et al., 1998; Tamaru et al., 2001) and it projects to the CNA as well as to the bed nucleus of the stria terminalis (BNST) which has direct connections to the hypothalamus and brainstem regions (Davis and Whalen, 2001) involved in the regulation of sleep (Sanford et al., 2002; Jones, 2005; Siegel, 2005; Tang et al., 2005; Sanford et al., 2006); thus, suggesting it may be an important region for mediating the effects of mGlu II receptors on sleep. To test this hypothesis, we microinjected into the BA various dosages of an mGlu II agonist (LY37) or antagonist (LY34) to determine whether it is a potential site for mediating their effects on sleep.

2. Results

2.1. Microinjection Sites

Microinjection sites for the rats used in this study are shown in Fig. 9. Although there was variation in the placement, the locations indicated that each injection of drugs infused into the BA. Diffusion to other amygdaloid nuclei may also have been possible.

2.2 Millimolar Dosage Range of LY37

This study examined sleep after microinjection into the BA of three millimolar concentrations of LY37 (3.2 mM, 5.3 mM or 10.7 mM; 0.5 μl) or vehicle alone (VEH;

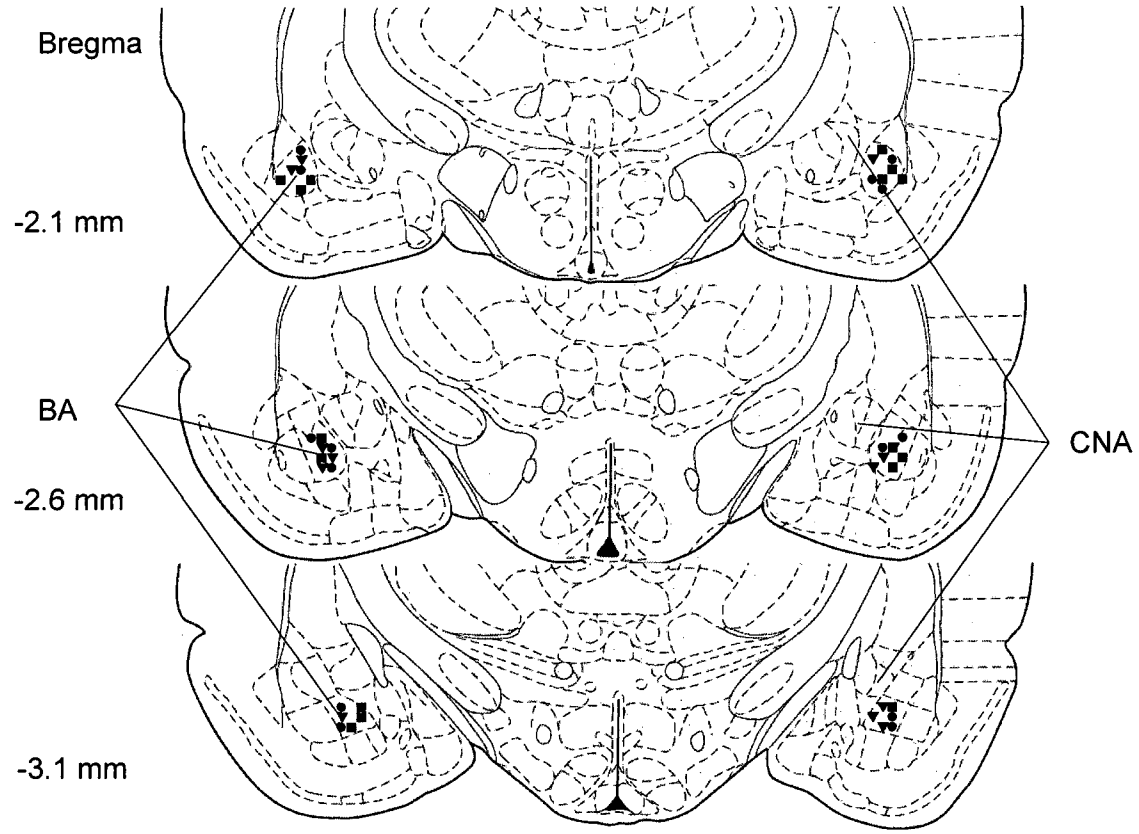


Fig. 9 - Line drawings of the amygdala showing placements of drug cannulae. Triangles indicate injection sites of 6 rats that received bilateral microinjections into the BA of three concentrations of LY37 (3.2 mM, 5.3 mM or 10.7 mM; 0.5 μl) and vehicle alone (VEH, 0.5 μl) control. Rectangles indicate injection sites of 8 rats that received bilateral microinjections into the BA of three concentrations of LY37 (0.1 nM, 2.0 nM or 10.0 nM; 0.5 μl) and a vehicle (VEH, 0.5 μl) control. Circles indicate injection sites of 7 rats that received bilateral microinjections into the BA of three concentrations of the antagonist, LY34 (1 nM, 30 nM or 60 nM; 0.5 μl) and a vehicle (VEH, 0.5 μl) control.

distilled water, 0.5 µl). Fig. 10 presents REM (Panel A) and NREM (Panel B) plotted as 2 h totals across the 20 h recording periods. For statistical analysis, the time spent in REM, NREM, total sleep and wakefulness was considered in 4 h blocks over the 20 h recording periods. The resulting data were analyzed by one-way repeated measures ANOVAs for each 4 h block.

REM

The ANOVAs for total REM amounts found significant drug effects for the first [$F(3, 15)=8.809$, $p<0.005$], second [$F(3, 15)=26.444$, $p<0.001$] and third [$F(3, 15)=6.174$, $p<0.01$] 4 h blocks. Compared to VEH, injection of LY37 at all three dosages significantly decreased REM in the first, second and third 4 h blocks (Table 1). There were no significant drug effects during the fourth or fifth 4 h block.

The analyses for REM episode numbers found a significant drug effect for the first [$F(3, 15)=11.972$, $p<0.001$], second [$F(3, 15)=19.790$, $p<0.001$] and third [$F(3, 15)=5.482$, $p<0.05$] 4 h blocks. The analyses were similar for REM episode durations with significant drug effects found for the first [$F(3, 15)=9.601$, $p<0.001$], second [$F(3, 15)=7.169$, $p<0.005$] and third [$F(3, 15)=7.684$, $p<0.005$] 4 h blocks. Both REM episode numbers and REM episode durations were significantly decreased at all dosages during the first and second 4 h block and at the low and medium dosage during the third 4 h block (Table 1). There were no significant drug effects during the fourth or fifth 4 h block.

NREM, Total Sleep and Wakefulness

The ANOVAs for total NREM amounts, total sleep and wakefulness did not reveal significant drug effects during any 4 h block. The ANOVAs for NREM episode numbers

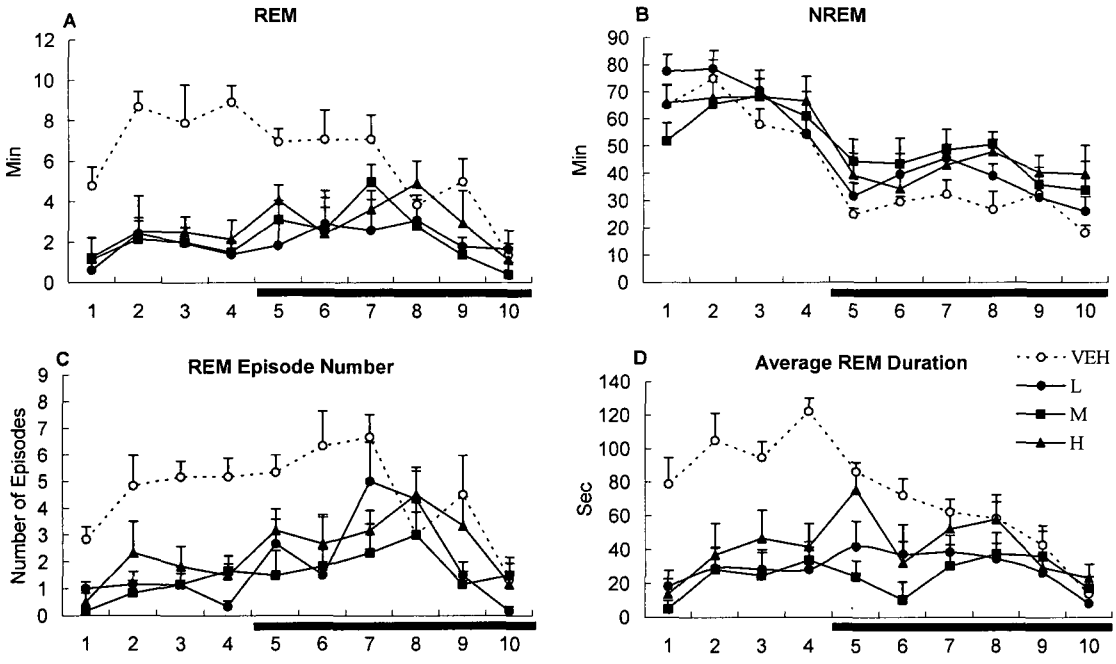


Fig. 10 - 2 h block plots (mean±SEM) of REM (A), NREM (B), REM Episode Numbers (C) and Average REM Durations (D) after microinjection of the mGlu II receptor agonist LY37 (L: 3.2 mM, M: 5.3 mM or H: 10.7 mM; 0.5 µl) or vehicle (VEH, 0.5 µl) into the BA.

Table 1 - Time spent (min) in REM, NREM, Total Sleep, Wakefulness, REM Episode Numbers and REM Average Durations (mean±SEM) during 4 h blocks after microinjection of LY37 (Low: 3.2 mM, Medium: 5.3 mM or High: 10.7 mM; 0.5 µl) or vehicle (VEH, 0.5 µl) into the BA.

Variable	VEH	Low	Medium	High
REM				
B1	13.5 (2.6)	3.8 (1.2)**	3.0 (1.2)**	3.3 (0.9)**
B2	16.8 (1.2)	4.6 (1.4)***	3.3 (1.1)***	3.5 (1.4)***
B3	14.0 (1.9)	7.2 (2.5)*	4.7 (2.8)*	5.7 (2.4)**
B4	10.9 (1.6)	8.4 (2.3)	5.8 (1.8)	7.8 (2.3)
B5	6.3 (2.1)	4.4 (0.8)	3.1 (0.4)	1.8 (0.5)
NREM				
B1	140.3 (10.4)	156.2 (11.2)	118.3 (21.1)	134.0 (7.7)
B2	112.3 (9.7)	125.3 (13.3)	126.1(10.3)	135.0 (12.9)
B3	54.6 (1.9)	71.3 (11.3)	79.2 (14.0)	73.8 (17.3)
B4	58.9 (10.9)	84.5 (6.9)	88.1 (16.1)	90.7 (10.4)
B5	50.5 (8.9)	57.2 (8.2)	72.1 (13.9)	79.8 (12.3)
Total Sleep				
B1	153.8(11.9)	159.4 (11.7)	121.3 (22.1)	137.7 (8.4)
B2	129.2 (10.7)	128.8 (13.6)	129.4 (10.2)	139.6 (12.2)
B3	68.6 (3.2)	77.0 (12.0)	84.0 (13.0)	81.0 (16.6)
B4	69.9 (12.3)	92.3 (7.5)	93.8 (16.1)	99.1 (9.1)
B5	56.8 (10.9)	58.9 (8.0)	75.2(13.7)	84.2 (12.1)
Wakefulness				
B1	86.3(11.9)	80.6 (11.7)	118.7 (22.1)	102.3 (8.4)
B2	110.8 (10.7)	111.2 (13.6)	110.6 (10.2)	100.4 (12.2)
B3	171.4 (3.2)	163.0 (12.0)	156.1 (13.0)	159.0 (16.6)
B4	170.1 (12.3)	147.7 (7.5)	146.2 (16.1)	99.1 (9.1)
B5	183.2 (10.9)	181.1 (8.0)	164.8 (13.7)	155.8 (12.1)
REM Episide Number				
B1	7.7 (1.3)	2.2 (0.6)**	1.0 (0.5)***	2.8 (1.3)**
B2	10.3 (1.3)	1.5 (0.5)***	2.8 (1.1)***	3.3 (1.0)***
B3	11.7 (1.7)	4.2 (1.8)*	3.3 (2.7)*	5.8 (1.9)
B4	9.7 (1.3)	9.3 (2.4)	5.3 (2.2)	7.7 (1.7)
B5	5.8 (2.0)	1.7 (0.5)	2.6 (0.8)	4.5 (1.1)
REM Average Duration				
B1	91.8 (14.7)	23.8 (8.9)**	16.7 (7.9)**	25.4 (13.2)**
B2	108.5 (15.6)	28.3 (10.8)*	29.2 (11.1)**	44.2 (13.7)**
B3	79.1 (6.8)	39.6 (15.0)*	17.2 (9.3)**	53.7 (13.5)
B4	60.3 (10.6)	36.5 (9.2)	33.9 (12.3)	55.1 (7.7)
B5	28.4 (7.1)	17.4 (7.1)	26.7 (7.3)	26.4 (7.2)

* $p<0.05$, ** $p<0.01$, *** $p<0.001$; significant differences relative to VEH. Comparisons were conducted using Tukey tests.

and average NREM episode durations also did not find any significant drug effect.

2.3. Nanomolar Dosage Range of LY37

Because we did not observe a dosage dependent effect on REM or other sleep wake states after microinjections into the BA of millimolar concentrations of LY37, we ran an additional experiment in which a separate group of rats (n=8) received microinjections of LY37 at the nanomolar level (0.1 nM, 2 nM or 10 nM; 0.5 µl). Analyses were conducted as described above for the higher dosage range.

REM

During the first 4 h block, the ANOVA for total REM amounts (Fig. 11A) found a significant drug effect [$F(3, 21)=5.478$, $p<0.01$]. Compared to VEH, injection of LY37 significantly decreased REM with injection at the high ($p<0.01$) and medium dosage ($p<0.05$). During the second 4 h block, the ANOVA for total REM amounts found a significant drug effect [$F(3, 21)=3.455$, $p<0.05$]. Compared to VEH, injection of LY37 significantly decreased REM with injection at the high dosage ($p<0.05$). There were no significant drug effects during any other 4 h block.

Fig. 12 presents REM average durations (Panel A) and episode numbers (Panel B) plotted as 4 h totals after microinjection into the BA of the various nanomolar dosages of LY37 or VEH. During the first 4 h block, the ANOVA for REM episode numbers found a significant drug effect [$F(3, 21)=5.090$, $p<0.01$]. Compared to VEH, injection of LY37 significantly decreased the REM episode number with injection at the high dosage ($p<0.01$). There were no significant drug effects during any other 4 h block. During the first 4 h block, the ANOVA for REM episode durations found a significant drug effect

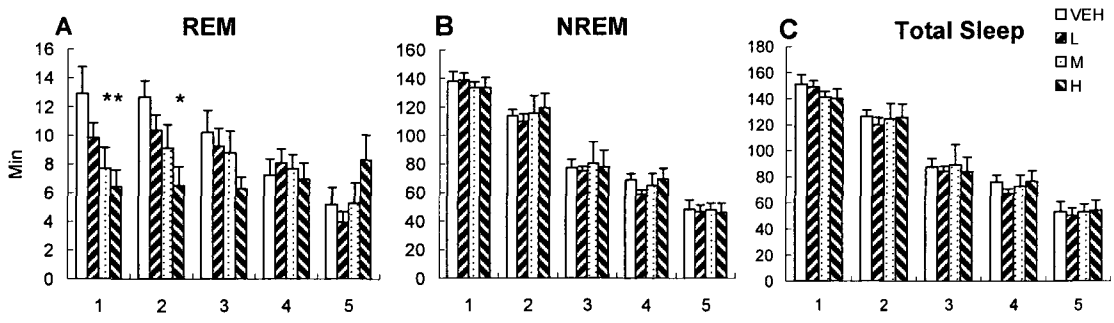


Fig. 11 - Time spent in REM (A), NREM (B) and Total Sleep (C) plotted in 4 h blocks for 20 h after microinjection of the mGlu II receptor agonist LY37 (L: 0.1 nM, M: 2 nM or H: 10 nM; 0.5 μl) or vehicle (VEH, 0.5 μl) into the BA. * $p<0.05$, significant differences relative to VEH. Comparisons were conducted using Tukey tests.

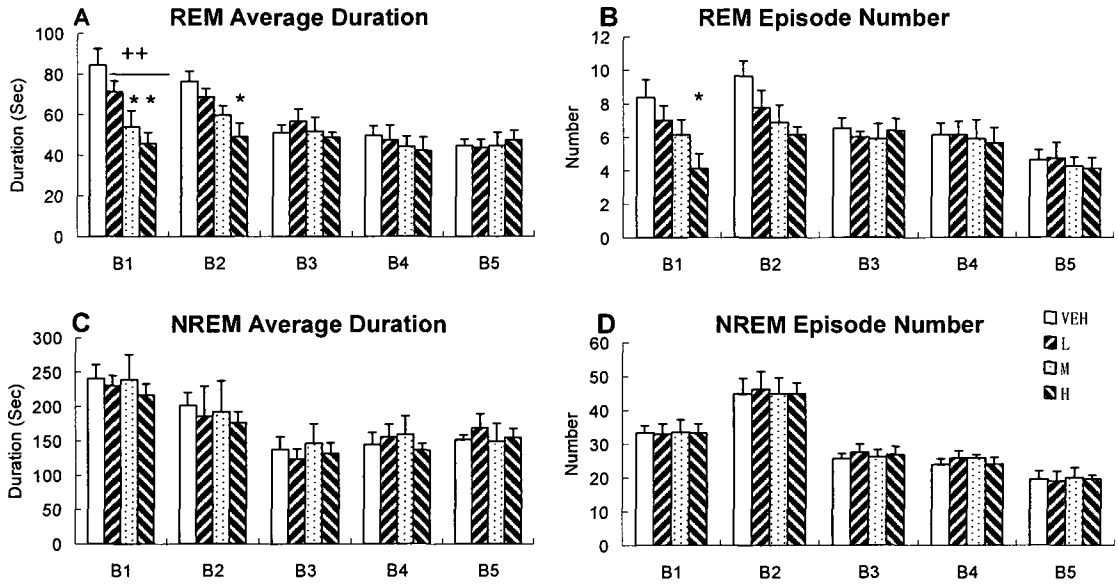


Fig. 12 - Plots of REM and NREM parameters in 4 h blocks (mean±SEM). REM Average Durations (A), REM Episode Numbers (B), NREM Average Durations (C) and NREM Episode Numbers (D) after microinjection of LY37 (L: 0.1 nM, M: 2 nM or H: 10 nM; 0.5 μl) or vehicle (VEH; 0.5 μl) into the BA.* $p<0.05$, significant differences relative to VEH; ++ $p<0.05$, significant differences between different drugs. Comparisons were conducted using Tukey tests.

[F(3, 15)=9.204, p<0.001]. Compared to VEH, injection of LY37 significantly decreased REM episode numbers at the high (p<0.001) and medium (p<0.01) dosages. There was also a significant difference between low dosage and high dosage (p<0.05). During the second 4 h block, the ANOVA for REM durations found a significant drug effect [F(3, 15)=5.413, p<0.01]. Compared to VEH, injection of LY37 significantly decreased the average REM duration at the high dosage (p<0.01). There were no significant drug effects during any other 4 h block.

NREM, Total Sleep and Wakefulness

As with the higher range of concentrations, the ANOVAs for NREM amounts (Fig. 11B), total sleep amounts (Fig. 11C) and wakefulness amounts did not reveal significant drug effects. There were no significant drug effects in the analyses for NREM average durations (Fig. 12C) or episode numbers (Fig. 12D).

2.4. Effects of LY34 on Sleep

Fig. 13 presents REM, NREM and total sleep plotted as 2 h totals after microinjection into the BA of different dosages of LY34 (L: 1 nM, M: 30 nM, H:60 nM; 0.5 μl) or VEH (0.01 M PBS, 0.5 μl) alone. The data for REM, NREM, total sleep and wakefulness are presented in Table 2.

REM

The ANOVAs for total REM amounts found significant drug effects for the first [F(3, 18)=6.549, p<0.01] and second [F(3, 18)=4.619, p<0.05] 4 h blocks. In the first 4 h block, injection of LY34 at the high dosage (60 nM) significantly decreased REM compared to that of animals injected with VEH (p<0.005) or the low dosage (p<0.05). In the second 4 h block, the high dosage significantly decreased REM compared to VEH (p<0.05). The

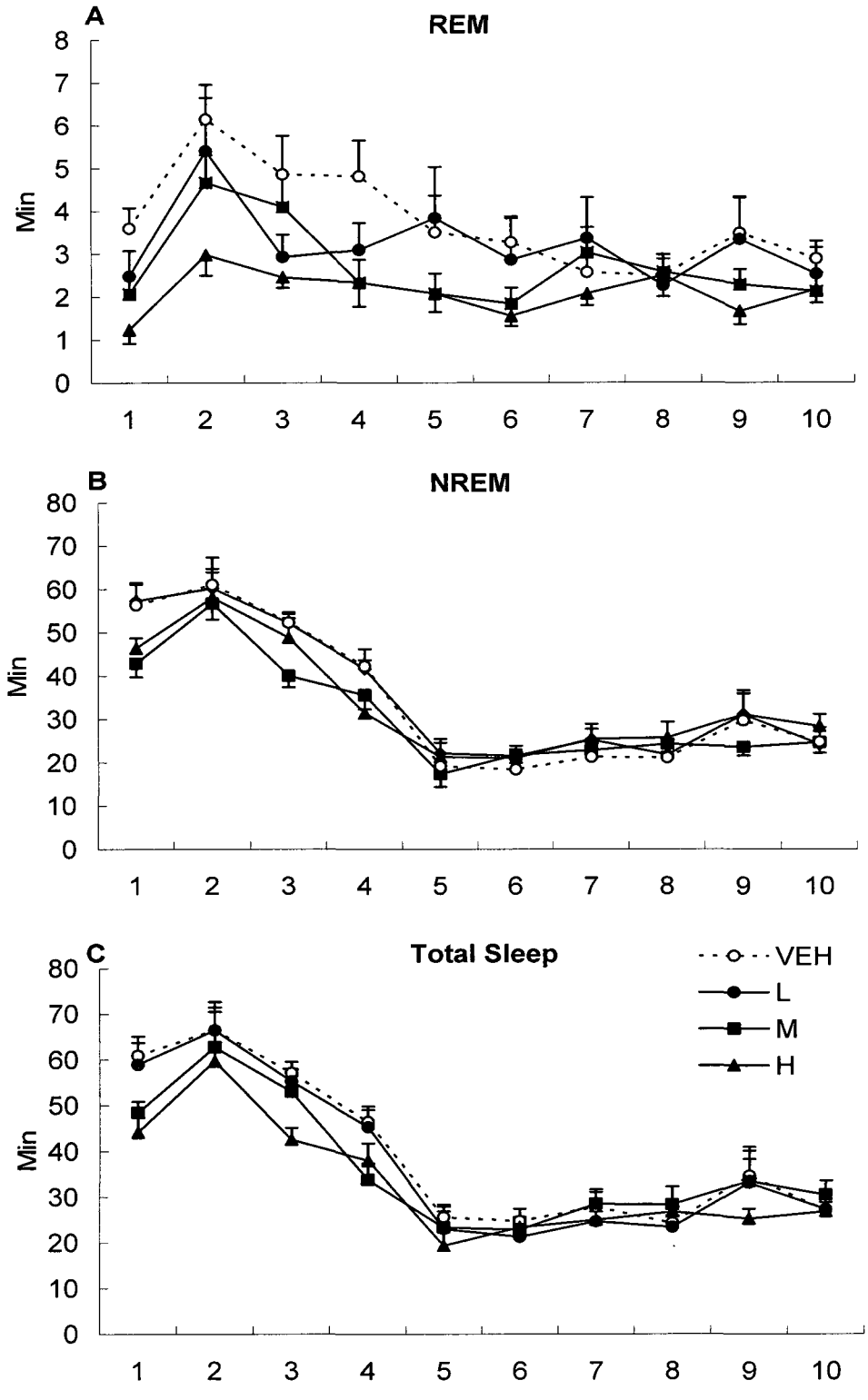


Fig. 13 - Time spent in REM (A), NREM (B) and Total Sleep (C) plotted in 2 h blocks for 20 h after microinjection of the mGlu II receptor antagonist LY34 (L:1 nM, M: 30 nM or H: 60 nM; 0.5 µl) or vehicle (VEH, 0.5 µl) into the BA.

Table 2 - REM, NREM, Total Sleep and Wakefulness during 4 h blocks after microinjection of LY34 (Low: 1 nM, Medium: 30 nM or High: 60 nM; 0.5 µl) or vehicle (VEH, 0.5 µl) into the BA.

Variable	VEH	Low	Medium	High
REM				
B1	9.7 (1.3)	7.8 (1.7)	6.7 (0.9)	4.2 (0.7)**
B2	9.6 (1.2)	6.0 (1.0)	6.4 (1.2)	4.8 (0.7)*
B3	6.8 (1.2)	6.7 (2.0)	3.9 (0.8)	3.6 (0.3)
B4	5.1 (0.7)	5.6 (1.5)	5.6 (0.7)	4.6 (0.7)
B5	6.4 (1.0)	5.9 (1.1)	4.4 (0.7)	3.8 (0.4)
NREM				
B1	117.5 (6.6)	117.4 (6.6)	104.5 (10.3)	99.6 (5.3)
B2	93.8 (3.0)	94.6 (5.6)	80.3 (7.3)	75.7 (5.2)**
B3	43.5 (3.7)	37.6 (4.7)	42.3 (4.1)	39.1 (3.7)
B4	47.0 (4.9)	42.4 (7.2)	51.2 (5.0)	47.1 (2.7)
B5	55.2 (5.4)	54.5 (8.7)	59.4 (6.2)	48.2 (3.6)
Total Sleep				
B1	127.2 (7.2)	125.3 (7.9)	111.2 (10.9)	103.9 (5.9)
B2	103.5 (4.0)	100.6 (6.5)	86.8 (8.1)	80.5 (5.4)**
B3	50.3 (3.9)	44.3 (6.4)	46.2 (4.7)	42.7 (3.9)
B4	52.0 (5.3)	48 (8.1)	56.8 (5.5)	51.7 (3.0)
B5	61.6 (6.1)	60.3 (9.5)	63.8 (6.5)	52 (3.8)
Wakefulness				
B1	112.8 (7.2)	114.7 (7.9)	128.8 (10.9)	136.1 (5.9)
B2	136.5 (4.0)	139.4 (6.5)	153.2 (8.1)	159.5 (5.4)**
B3	189.7 (3.9)	195.7 (6.4)	193.8 (4.7)	197.3 (3.9)
B4	188.0 (5.3)	192.0 (8.1)	183.2 (5.5)	188.3 (3.0)
B5	178.4 (6.1)	179.7 (9.5)	176.2 (6.5)	188 (3.8)

* $p<0.05$, ** $p<0.01$, *** $p<0.001$; significant differences relative to VEH. Comparisons were conducted using Tukey tests.

analyses of total REM amounts did not reveal any significant drug effects in any other 4 h block.

The ANOVA for REM episode numbers found a significant drug effect for the second 4 h block [$F(3, 18)=5.484$, $p<0.01$]. Injection of LY34 at the high dosage significantly decreased the REM episode number compared to VEH ($p<0.01$). During the first 4 h block, the ANOVA for average REM durations found a significant drug effect [$F(3, 18)=4.156$, $p<0.05$]. Injection of LY34 at the high dosage significantly decreased the average REM duration compared to VEH ($p<0.05$). There were no other significant effects found for REM episode numbers or REM durations.

NREM, Total Sleep and Wakefulness

The ANOVA for total NREM amounts found a significant drug effect in the second 4 h block [$F(3, 18)=7.355$, $p<0.01$]. The high dosage of LY34 significantly decreased NREM compared to that of animals injected with vehicle ($p<0.01$) or low dosage ($p<0.01$). There were no significant alterations in total NREM and there were no significant differences in the analyses for NREM episode numbers or average NREM durations in any of the 4 h blocks.

During the second 4 h block, the ANOVAs revealed significant drug effects for total sleep amounts [$F(3, 18)=7.442$, $p<0.01$] and total wakefulness amounts [$F(3, 18)=7.442$, $p<0.01$]. The high dosage of LY34 significantly decreased total sleep compared to VEH ($p<0.01$) or the low dosage of LY34 ($p<0.05$). Parallel changes were found in wakefulness in comparisons of the high dosage of LY34 to VEH ($p<0.01$) and the low dosage of LY34 ($p<0.05$). No other comparisons were significant.

3. Discussion

Microinjection of the mGlu II agonist, LY37, into the BA at both nanomolar and millimolar concentrations significantly decreased REM without significantly altering NREM, total sleep or wakefulness. By comparison, microinjection of a high dosage of the specific mGlu II antagonist, LY34, into the BA significantly suppressed REM, NREM and total sleep and increased wakefulness. These results demonstrate that the selective activation of mGlu II receptors in the BA significantly and specifically suppresses REM in the rat whereas blocking mGlu II receptors can decrease sleep and enhance wakefulness. Further, these results suggest that the BA plays a role in the regulation of REM and NREM as well as the overall arousal level. These data are consistent with work of other researchers demonstrating that systemic injection of mGlu II agonists, LY37 or LY35, suppresses REM in a dosage-dependent manner (Feinberg et al., 2002; Ahnaou et al., 2009) whereas injection of LY34 increases arousal at the expense of REM and NREM (Feinberg et al., 2005). The present results for the first time demonstrate a specific brain area that could be a site where mGlu II receptors exert their effects on sleep.

The injection of LY34 at the highest concentration (60 nM) we tested significantly increased wakefulness at the expense of REM, NREM and total sleep amounts. These results may seem surprising as microinjection of the agonist selectively decreased REM alone. However, the results are consistent with the theoretical role of mGlu II receptors in glutamate release (Cartmell and Schoepp, 2000). mGlu II receptors may serve as glutamate release autoreceptors in the presynaptic remote area and blockade of these receptors with their antagonist may contribute to the shift of glutamate release to a higher level which may increase the activity level in the BA. The increase of the BA activity

may be the reason for increasing arousal level and suppressing sleep. The observation of mGlu II receptors located in the perisynaptic remote area in the presynaptic terminal (Yokoi et al., 1996) suggests that these receptors may only work under conditions of high glutamate release as the released glutamate has to reach a level sufficient to travel to the sites of mGlu II receptors that are far away from the glutamate release site. This hypothesis is supported by the need for increased glutamate release for the activation of mGlu II receptors (Scanziani et al., 1997). It has been shown that under repetitive stimulation hippocampal mossy fiber synapses increase glutamate release and glutamate concentration in the cleft to allow glutamate to spread away from the synapse and therefore activate mGlu II receptors. The activated mGlu II receptors will subsequently suppress glutamate release. This theory gains further support by the fact that application of mGlu receptor antagonist α-methyl-4-carboxyphenylglycine (MCPG) enhances excitatory postsynaptic currents (EPSC) induced by repetitive stimuli (Scanziani et al., 1997).

Interestingly, mGlu II agonists may not alter the basal glutamate release suggesting that other mechanisms may be responsible for their selective suppression of REM. This suggestion is consistent with the finding that systemic or local administration of LY37 reverses ketamine-evoked glutamate release in the medial prefrontal cortex (mPFC) but does not alter the basal glutamate level (Lorrain et al., 2003). Moreover, systemic or local administration of LY37 significantly decreases the GABA level in the hippocampus (Smolders et al., 2004). Biochemistry studies further suggest that mGlu receptor agonists suppress GABA release from neurons in several brain areas, including the hippocampus, the thalamus and the accessory olfactory bulb (Anwyl, 1999; Cartmell and Schoepp,

2000). Therefore, we suggest that injection of LY37 did not change the basal glutamate release level but may have suppressed GABA release in the BA and altered the regulation of the CNA.

The CNA has long been implicated in the regulation of REM. Pharmacologic administration of various receptor agents into the CNA has direct effects on REM (Sanford et al., 1995; Calvo et al., 1996). Inactivation of the CNA by muscimol, a $GABA_A$ agonist, decreases REM without altering NREM whereas microinjection of $GABA_A$ antagonist bicuculline into the CNA increases REM (Sanford et al., 2002). Temporal inactivation of the CNA by tetrodotoxin (TTX), a sodium channel blocker, significantly suppresses REM and reduces arousal as indicated by shortened NREM latency and decreased activity in an arousing environment (Tang et al., 2005). In addition, microinjection of the cholinergic agonist, carbachol, into the CNA of rats suppresses REM (Sanford et al., 2006). The CNA has direct projections to brainstem areas involved in the generation and regulation of REM, including the locus coeruleus, the laterodorsal tegmental nucleus and the dorsal raphe nucleus (Krettek and Price, 1978; Takeuchi et al., 1982; Petrov et al., 1994). Considering these neuroanatomical projections and its connection with other amygdaloid nuclei, it is clear that the amygdala may exert its regulation on REM through the CNA.

The BA appears to be involved in the amygdalar regulation of sleep. Histochemical tracer studies demonstrate that the BA receives various cortical and subcortical sensory inputs from the mPFC, the hippocampus, the midbrain and pons (Pitkanen, 2000). It has been demonstrated that the BA projects to the CNA and the BNST (Pitkanen, 2000). Various evidence indicates that the BA and CNA are essential for processing and

acquiring conditioned fear as lesions of either area may disrupt conditioned fear (Sarter and Markowitsch, 1985; Davis, 1997; Gale et al., 2004). Furthermore, the amygdala appears to be a critical emotional modulator of sleep (Morrison et al., 2000; Sanford et al., 2002; Tang et al., 2005; Sanford et al., 2006). Many studies have focused on exploring the role of the CNA in the regulation of sleep and most techniques involved include electrical or chemical lesion, electrical stimulation and the local infusion of various drugs. However, these methods may influence projections that go through the CNA. For example, TTX can suppress not only neuronal cells in the CNA but also the projections passing through the CNA from the BA to the BNST (Davis and Whalen, 2001; Tang et al., 2005). Therefore, activation or inactivation of the CNA may disrupt the projections from the BA passing through the CNA. In addition, the BNST also has many projections to the hypothalamus and the brain stem, thus allowing the BA to activate the same targets through the BNST as it does through the CNA (Davis and Whalen, 2001).

The BA may be in a position to link input signals with outputs that regulates sleep. Two types of neurons are found in the BA. The first type, pyramidal neurons, comprises the majority of the total neuronal population. These neurons demonstrate a high level of immunoreactivity for glutamate and are considered glutamatergic neurons (Smith and Pare, 1994). Pyramidal neurons receive most afferent inputs from cortical and subcortical structures and send output projections (Farb et al., 1995). The second type of neurons are interneurons which have smaller somata compared to pyramidal neurons (McDonald, 1992). Interneurons in the BA receive excitatory inputs from cortical, thalamic and local sources (Nitecka and Ben-Ari, 1987). These neurons are GABAergic and serve as local circuit interneurons (Pitkanen and Amaral, 1994). Stimulation of these neurons produces

inhibitory synaptic potentials (Rainnie et al., 1991). Interneurons send inhibitory projections to pyramidal neurons, which in turn send excitatory projections to intercalated cells (Pitkanen, 2000; Sah et al., 2003). Intercalated cells are small GABAergic cell bodies located between the BLA and the CNA (Millhouse, 1986). It has been found that intercalated cell bodies contain a population of neurons firing at much higher rates than cells in neighboring amygdaloid nuclei. Moreover, some individual intercalated neurons show state-dependent changes in firing rates (Collins and Pare, 1999). These data suggest that intercalated cells may modulate neurons in their projection sites through tonic inhibitory inputs and that this modulation may be altered by those intercalated cells that show state-dependent firing rates. The intercalated cell bodies are reported to receive excitatory inputs from the BLA and thus generate inhibitory effects in the neurons of the CNA (Royer et al., 1999).

Based on the circuitry of the BA, we propose a model to explain the effects induced by LY37 (Fig. 14). In this model, infusion of LY37 into the BA suppresses the release of GABA that would, in turn, decrease interneuron activity. The pyramidal neurons in the BA thus receive decreased inhibitory inputs from interneurons and in turn send increased excitatory outputs to intercalated neurons. The result would be an increase in inhibitory signals to neurons in the CNA, thereby suppressing CNA activity and decreasing REM. This hypothesis is consistent with studies that found suppressing the CNA activity decreases REM (Sanford et al., 2002; Sanford et al., 2006).

In the current study we found that mGlu II receptors in the BA play a role in the regulation of sleep. Furthermore, it has been well documented that the BA has a role in

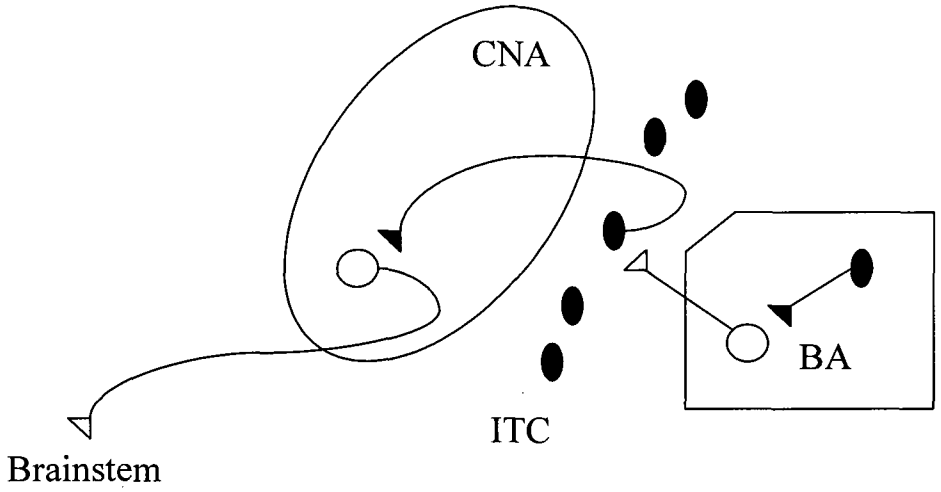


Fig. 14 - Model for the mechanism of the BA regulation of REM through its association with intercalated cells and the CNA. Interneurons in the BA send inhibitory projections to pyramidal cells which in turn send excitatory projections to intercalated cells. Intercalated cells send inhibitory projections to neurons in the CNA, which have connections with brainstem areas. ITC: intercalated cells. GABAergic (inhibitory) neurons are depicted with filled symbols; Glutamatergic (excitatory) neurons are depicted with open symbols.

the acquisition and expression of conditioned fear (Ono et al., 1995; Yaniv and Richter-Levin, 2000; Gale et al., 2004) as well as in the extinction of fear (Myers and Davis, 2007; Quirk and Mueller, 2008). mGlu II receptors have been reported to be involved in the development of stress and anxiety (Palucha and Pilc, 2007). The anxiolytic property of mGlu II agonists has been supported by behavioral studies. For example, systemic administration of LY37 attenuates the increase of norepinephrine levels in the prefrontal cortex in rats exposed to an elevated platform (Lorrain et al., 2005). Oral administration of the agonist, LY354740 (LY35), increases open-arm activity in the elevated plus maze in mice (Monn et al., 1997). In contrast, the antagonist, LY34 has anxiogenic properties as intraperitoneal injections of LY34 increase plasma corticosterone in mice (Scaccianoce et al., 2003). These data also suggest that mGlu II receptors may play a role in the stress response. Furthermore, sleep and anxiety disorders are associated, with patients of anxiety disorders often suffering from sleep disturbances (Benca et al., 1992). Therefore, investigation in the role of mGlu II receptors in the regulation of sleep and stress may provide future approaches for the treatment of anxiety disorders.

In summary, the role of mGlu II receptors in the regulation of sleep has only been minimally explored. The present data indicate that the BA may be a brain region where these receptors may exert their influence on sleep. Considering that the BA is an essential site in contextual fear conditioning and fear extinction, work is needed to determine if the BA may also play a role in the regulation of stress-induced alterations on sleep.

4. Experimental Procedure

4.1. Subjects

The subjects were 21 male Wistar rats obtained from Harlan (Indianapolis, IN). The rats were approximately 300 gm at the time of surgery. The rats were individually housed in polycarbonate cages and given ad lib access to food and water. The room for sleep recording/housing was kept on a 12:12 light:dark cycle with lights on from 7:00 AM to 19:00 PM. The room temperature was maintained at 24.5° ± 0.5°C.

4.2. Surgery

One week following their arrival, the rats were implanted with skull screw electrodes for recording the electroencephalogram (EEG) and with stainless steel wire electrodes sutured into the nuchal muscles for recording the electromyogram (EMG). Leads from the implanted electrodes were routed to a 9-pin miniature plug attached to the skull. Guide cannulae (26 gauge) for microinjections were bilaterally implanted to introduce the tip of the injection cannulae into the BA (AP=-2.6, DV=8.0, ML= ±4.8 mm (Paxinos G, 1997)). All surgeries were conducted under aseptic conditions. The rats were anesthetized with isoflurane (5% induction; 2% maintenance). Ibuprofen (15 mg/kg) was available in water 24 h before surgery and 3 days after surgery to alleviate pain. All procedures were conducted in accordance with the National Institutes of Health *Guide for the Care and Use of Experimental Animals* and were approved by Eastern Virginia Medical School's Animal Care and Use Committee (Protocol #07-005).

4.3. Drug Preparation

Both the agonist (LY37) and antagonist (LY34) were purchased from Tocris Bioscience (Ellisville, MO). Immediately prior to the microinjection, LY37 was dissolved in distilled water which served as vehicle. LY34 was dissolved in 0.01 M phosphate buffered saline (PBS) which served as vehicle.

4.4. Microinjection Procedures

After surgery, the rats were allowed 14 days for recovery. Then they were habituated to the microinjection procedures for two days. After each habituation handling session, the rats were connected to cables for 20 h sleep recording.

After habituation, 6 rats received bilateral microinjections into the BA of three concentrations of LY37 (3.2 mM, 5.3 mM or 10.7 mM; 0.5 μl) or vehicle alone (0.5 μl) control. In experiments with LY37 administrated at nanomolar levels, 8 rats received bilateral microinjections into the BA of three concentrations of LY37 (0.1 nM, 2.0 nM or 10.0 nM; 0.5 μl) or a vehicle (0.5 μl) control. Similarly, 7 rats received bilateral microinjections into the BA of three concentrations of the antagonist, LY34 (1 nM, 30 nM or 60 nM; 0.5 μl) or a vehicle (0.5 μl) control. For each drug, microinjections were administrated in a counterbalanced order at 5-day intervals. Following each microinjection, the rats were returned to their home cages and connected to recording cables. Sleep was then recorded for 20 hours.

For microinjections, cannulae (33 ga.), which projected 1.0 mm beyond the tip of the guide cannulae, were secured in place within the guide cannulae. The injection cannulae were connected to lengths of polyethylene tubing that in turn were connected to 5.0 μl Hamilton syringes. The injection cannulae and tubing had been pre-filled with the solution to be injected. Solutions in a volume of 0.5 μl were slowly infused over 3 min, with the cannulae left in place 1 min prior to the start and 1 min after the completion of the microinjection.

4.5. Sleep Recording

Sleep recording was performed with rats in their home cages. A lightweight, shielded cable was connected to a plug in the rats' head. The cable was connected to a swivel that allowed free movement for the rats within their cages. EEG and EMG signals were processed by a Grass Model 12 polygraph equipped with model 12A5 amplifier and routed to an A/D board (Eagle PC30) housed in a Pentium class PC. The signal were digitized at 128 HZ and collected in 10-second epochs using a custom sleep data collection program.

4.6. Determination of Sleep

The EEG and EMG records were visually scored by a trained observer to determine wakefulness, NREM and REM in 10-second epochs (Sanford et al., 2002). Wakefulness was determined by the presence of low-voltage, fast EEG; high-amplitude, tonic EMG levels; and phasic EMG bursts that could be associated with gross body movements. NREM was scored by the presence of spindles interspersed with slow waves, lower muscle tone, and no gross body movements. REM was determined by the presence of low-voltage, fast EEG, theta rhythm, and muscle atonia.

4.7. Data Analysis

Statistical analyses were conducted using SigmaStat (SPSS, Inc., Chicago, Illinois). One-way repeated measures ANOVAs across drug treatment were used to analyze sleep alteration in 4 h blocks. The Tukey tests were used when all pairwise comparisons among means were made across drug treatments.

CHAPTER III

MICROINJECTION OF GROUP II METABOTROPIC GLUTAMATE RECEPTOR ANTAGONIST, LY341495, INTO THE BASAL AMYGDALA FACILITATES FEAR EXTINCTION AND ATTENUATES FEAR-INDUCED ALTERATIONS IN SLEEP

1. Introduction

In contextual fear conditioning, animals make associations between an aversive, fear-inducing stimulus, usually footshock, and the environment in which it is received. Afterwards, the animals exhibit fear responses when they experience the context alone. In addition to well established behavioral signs of fear (e.g., freezing defined as no movement except for respiration (Blanchard and Blanchard, 1969; Doyere et al., 2000)), sleep in the period after contextual fear is characterized by significant reductions in rapid eye movement sleep (REM) (Sanford et al., 2003; Tang et al., 2005). Repeated or prolonged exposure to fearful contexts without footshock will result in fear extinction in which the context no longer elicits behavioral fear responses (Myers and Davis, 2007; Quirk and Mueller, 2008). Sleep also normalizes after contextual fear extinction (Wellman et al., 2008).

The amygdala is essential in the regulation and expression of emotion and fear. Lesions of the basolateral complex of the amygdala (BLA) disrupt the acquisition and expression of fear conditioning (Maren et al., 1996). However, the BLA is not a unitary structure as it includes the lateral amygdala (LA), the basal amygdala (BA) and the accessory basal (AB) nuclei. Of these subregions of the BLA, the LA appears to be

primarily associated with auditory fear conditioning (LeDoux et al., 1990) whereas the BA is associated with contextual cues in fear conditioning (Fanselow and LeDoux, 1999; LeDoux, 2000). The BA receives projections from the ventral hippocampus (Canteras and Swanson, 1992), and electrolytic lesions in either site impairs conditioned fear to contextual cues (Maren and Fanselow, 1995). Neurons in the BA are found to respond to conditioned and unconditioned stimuli, suggesting that the BA is the location where conditioned stimuli (CS) and unconditioned stimuli (US) are associated (Ono et al., 1995; Toyomitsu et al., 2002). In addition, ibotenate lesions of the BA disrupt contextual fear conditioning but not auditory fear conditioning (Onishi BK, 2009). The BA is also implicated in fear extinction. Inhibition of N-methyl-D-aspartate (NMDA) receptors in the BA disrupts the expression and extinction of conditioned fear to contextual stimuli (Lee and Kim, 1998).

The BA receives various sensory inputs and projects to the central nucleus of amygdala (CNA), which in turn projects to sleep related structures such as the brainstem, the basal forebrain and the preoptic nucleus of the hypothalamus (Pitkanen, 2000; Davis and Whalen, 2001). Research on the role of the amygdala and sleep has typically focused on the CNA. For example, microinjections into the CNA of GABAergic (Sanford et al., 2002), cholinergic (Sanford et al., 2006) and serotonergic (Sanford et al., 1995) agents significantly alter REM in rats. Indeed, most data indicate that the CNA plays an important role in the regulation of REM. In contrast, few studies have examined the potential role of the BA in the regulation of sleep.

In the BA, pyramidal neurons are the major neurons, which use glutamate as the primary neurotransmitter (Washburn and Moises, 1992). Group II metabotropic glutamate

(mGlu II) receptors, which are expressed in the BA, serve as autoreceptors for glutamate release and may play a modulatory role for determining glutamate level in the BA (Petralia et al., 1996). Evidence shows that systemic injection of the mGlu II receptor antagonist, LY341495 (LY34), induces significant c-Fos expression, a marker of neural activity, all over the brain (Linden et al., 2005), which suggests that blockage of mGlu II receptors may contribute to increased neuronal activity. Our previous studies have revealed that the microinjection of LY34 into the BA significantly increases wakefulness at the expense of REM, NREM and total sleep amounts. These data indicate that injection of LY34 into the BA may change the activity of the BA as well as subsequent sleep.

The involvement of the BA in conditioned fear and fear extinction also suggests a possible role of mGlu II receptors in the BA in fear extinction and its effects on sleep. Therefore, the purpose of this study was to investigate the role of mGlu II receptors in the BA in fear extinction and to determine whether any potential alteration in extinction was related to subsequent changes in sleep. Rats were trained in contextual fear using a footshock stressor and received LY34 (30 nM) or vehicle (VEH, 0.01 M PBS) prior to re-exposure to the fearful context alone on the following day (extinction training day). The rats were also tested in the context on the second day after shock training (test day) and at the ninth day after shock training (retest day). Freezing was examined as a measure of fear memory and sleep was recorded for 20 h after each session.

2. Results

2.1. Microinjection Sites

Microinjection sites for each rat used in the study are shown in Fig. 15. Although

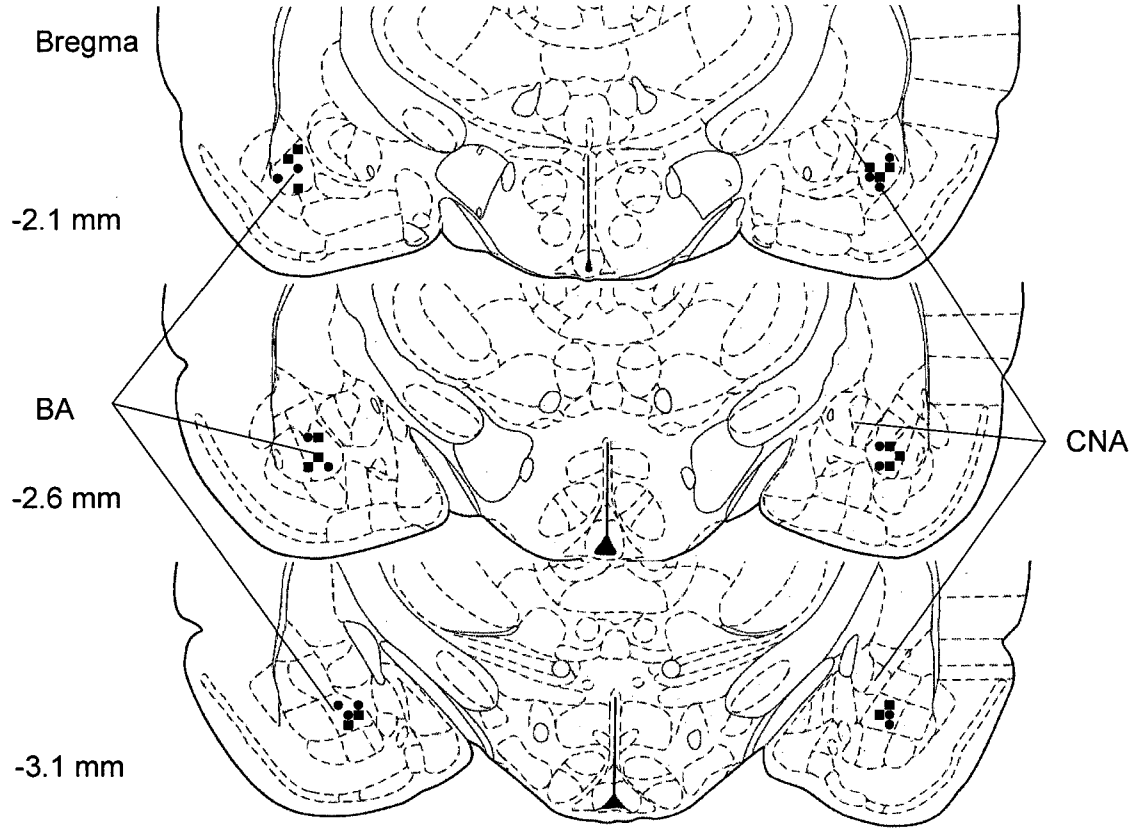


Fig. 15 - Line drawings of the amygdala showing placements of drug cannulae. Rectangles indicate injection sites of 8 rats that received bilateral microinjections into the BA of LY34 at 30 nM (0.5 μl). Circles indicate injection sites of 7 rats that received bilateral microinjections into the BA of vehicle (0.5 μl) control.

there was variation in the placement around the BA, the locations indicated that each injection of drugs was infused into the BA. Diffusion to other amygdaloid nuclei may also have been possible.

2.2. Freezing

The percentage of time spent in freezing (FT%) was calculated for specific periods during the shock training day, the extinction training day and the test and retest days. For shock training, the FT% data were divided into 3 periods: 5 min pre-shock period, 20 min shock training period and 5 min post-shock period. For the 1 h extinction training session, the data were divided into four blocks: P1 (1^{st} 15 min), P2 (2^{nd} 15 min), P3 (3^{rd} 15 min) and P4 (4^{th} 15 min) periods. The 30 min test and retest sessions were divided into two 15 min periods: P1 and P2. FT% was averaged for each minute during each time block. Freezing in the pre-shock period was used as a baseline to determine whether the animals showed significant fear responses during subsequent periods.

Freezing on Shock Training Day (Fig. 16A)

The ANOVA for freezing across periods and conditions found a significant main effect for the assessment period [$F(2, 2)=59.595$, $p<0.001$]. There were no significant differences in the two groups prior to being treated with VEH or LY34. Both groups of animals showed minimal freezing during the pre-shock period on the first day whereas FT% was significantly increased during the shock training session ($p < 0.001$) and the post-shock session ($p <0.001$) when compared to the pre-shock session.

Freezing during Extinction (Fig. 16B)

The ANOVA for freezing found a significant main effect for the drug [$F(1, 4)=7.892$, $p<0.01$] and a significant main effect for the assessment period [$F(4, 4)=108.878$,

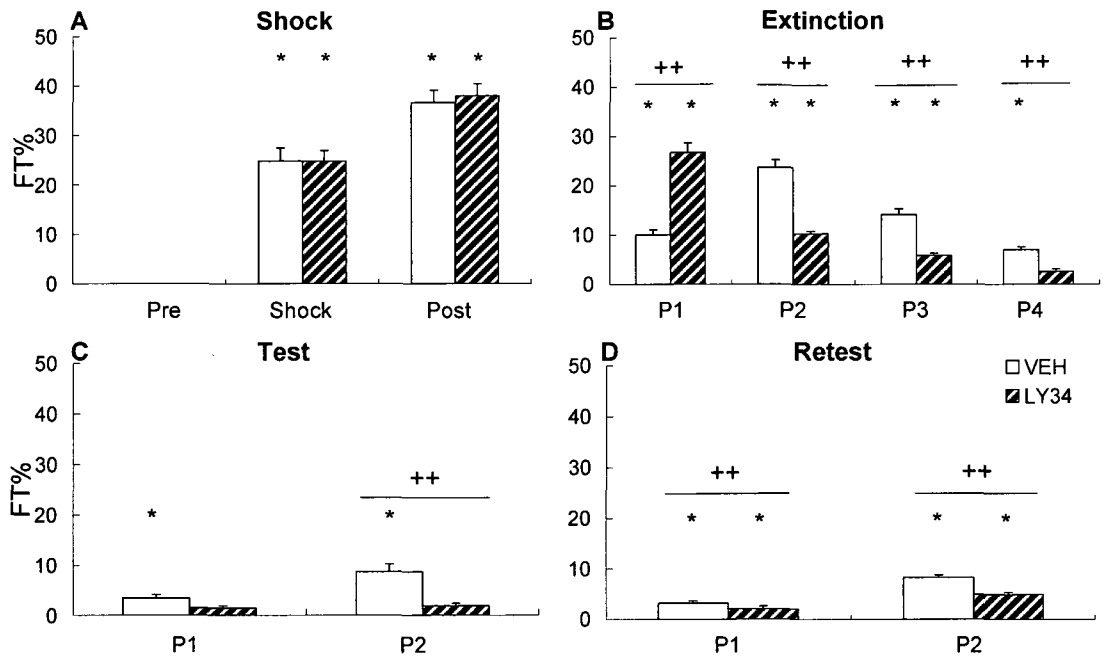


Fig. 16 - Average percentage of time spent in freezing (FT%) plotted for each measurement period. (A) FT% during pre-shock, shock and post-shock periods of vehicle injected animals (VEH) or LY34 injected animals (LY34) on the shock training day. (B) FT% during the P1 (1st 15 min), P2 (2nd 15 min), P3 (3rd 15 min) and P4 (4th 15 min) periods of extinction training. (C) and (D) FT% in the P1 (1st 15 min) and P2 (2nd 15 min) periods during test and retest session. * $p<0.05$, differences relative to pre-shock period of FT%; ++ $p<0.05$, differences between the VEH group and the LY34 group. Comparisons were conducted using Tukey tests.

$p<0.001$] as well as a significant interaction of drug treatment X assessment period [$F(4, 40)=58.985$, $P<0.001$]. During extinction training, VEH treated animals exhibited significantly increased FT% in P1 ($p<0.001$), P2 ($p<0.001$), P3 ($p<0.001$) and P4 ($p<0.001$) compared to pre-shock. VEH treated animals also exhibited increased FT% in P2 ($p<0.001$) compared to P1, decreased FT% in P3 compared to P2 ($p<0.001$) and decreased FT% in P4 compared to P3 ($p<0.001$).

Compared to pre-shock, LY34 treated animals showed significantly increased FT% in P1 ($p<0.001$), P2 ($p<0.001$) and P3 ($p<0.01$) whereas in P4 ($p=0.436$) FT% did not significantly differ from pre-shock levels. FT% was also significantly higher in P1 than in P2 ($p<0.001$), P3 ($p<0.001$) and P4 ($p<0.001$) and was also significantly higher in P2 than in P4 ($p<0.001$).

Compared to VEH treated rats, LY34 treated rats showed significantly greater FT% in P1 ($p<0.001$) and significantly decreased FT% during P2 ($p<0.001$), P3 ($p<0.001$) and P4 ($p<0.01$).

Freezing on Test Day (Fig. 16C)

The ANOVA for freezing found a significant main effect for the drug treatment [$F(1, 2)=23.731$, $p<0.001$] and a significant main effect for the assessment period [$F(2, 2)=26.010$, $p<0.001$] as well as a significant interaction of drug treatment X assessment period [$F(2, 24)=11.277$, $p<0.001$]. In VEH treated rats, FT% was significantly higher compared to pre-shock during P1 ($p<0.01$) and P2 ($p<0.001$). In LY34 treated rats, FT% did not significantly differ compared to pre-shock during either P1 or P2.

VEH and LY34 treated rats did not significantly differ in FT% during P1 ($p=0.069$); however, LY34 injected animals showed significantly lower FT% during P2 ($p<0.001$).

Freezing on Retest Day (Fig. 16D)

The ANOVA for freezing found a significant main effect for the drug treatment [$F(1, 2)=25.589$, $P<0.001$] and a significant main effect for the assessment period [$F(2, 2)=165.729$, $P<0.001$] as well as a significant interaction of drug treatment X assessment period [$F(2, 24)=11.281$, $p<0.001$].

In both VEH and LY34 injected animals, FT% during P1 and P2 was significantly higher than pre-shock ($p<0.001$). There was a significant difference between groups during either P1 ($p<0.05$) or P2 ($p<0.001$).

2.3. Sleep following each Training Session

REM, NREM and total sleep were analyzed in 4 h blocks for the 20 h recording periods during each phase of the experiment (shock training day, extinction day, test day and retest day). This resulted in five 4 h blocks: B1 and B2 in the light period and B3, B4 and B5 in the dark period. The ANOVA analysis for baseline sleep did not find significant differences between the two groups of animals. Therefore, the combined baseline data were used to determine whether the drug or treatments had effects on subsequent sleep. All the data were analyzed by two-way ANOVA.

Sleep on the Shock Training Day

The ANOVA for total REM amounts across treatments and time blocks found a significant main effect for the treatment [$F(2, 4)=20.296$, $p<0.001$] and a significant interaction for treatment X time block [$F(4, 8)=2.505$, $p<0.05$]. Compared to the baseline, shock training significantly suppressed REM in both groups during the first and second 4 h blocks (Fig. 17A , $p<0.01$). Shock training did not produce any significant change in REM between the two groups of animals (Fig. 17A) in any of the five 4 h blocks.

The ANOVA for total NREM amounts across treatments and time blocks found a significant main effect for the treatment [$F(2, 4)=5.444$, $p<0.01$] and a significant interaction for treatment X time block [$F(4, 8)=3.747$, $p<0.001$]. Shock training significantly suppressed NREM in both groups during the first 4 h block (Fig. 18A, $p<0.001$). However, VEH treated animals and LY34 treated animals showed no significant difference in NREM (Fig. 18A) in any of the five 4 h blocks.

The ANOVA for total sleep amounts across treatments and time blocks found a significant main effect for the treatment [$F(2, 4)=7.989$, $p<0.001$] and a significant interaction of treatment X time block [$F(4, 8)=3.952$, $p<0.001$]. Shock training significantly suppressed total sleep in both groups during the first 4 h block (Fig. 19A, $p<0.001$). However, VEH treated animals and LY34 treated animals showed no significant difference in total sleep (Fig. 19A) in any of the five 4 h blocks.

Table 3 presents REM episode numbers and average durations during the 4 h blocks following each treatment. The ANOVA for REM episode numbers during the shock training day found a significant main effect for shock [$F(2, 4)=52.602$, $p<0.001$] and a significant interaction of treatment X time block [$F(4, 8)=12.117$, $p<0.001$]. Shock significantly suppressed REM episode numbers for both groups during the first and second 4 h blocks (Table 3, $p<0.001$). Also the ANOVA for REM average durations found a significant main effect for shock [$F(2, 4)=12.740$, $p<0.001$] and a significant interaction of treatment X time block [$F(4, 8)=2.242$, $p<0.05$]. Shock significantly suppressed REM average durations for both groups during the first and second 4 h blocks (Table 3, $P<0.01$).

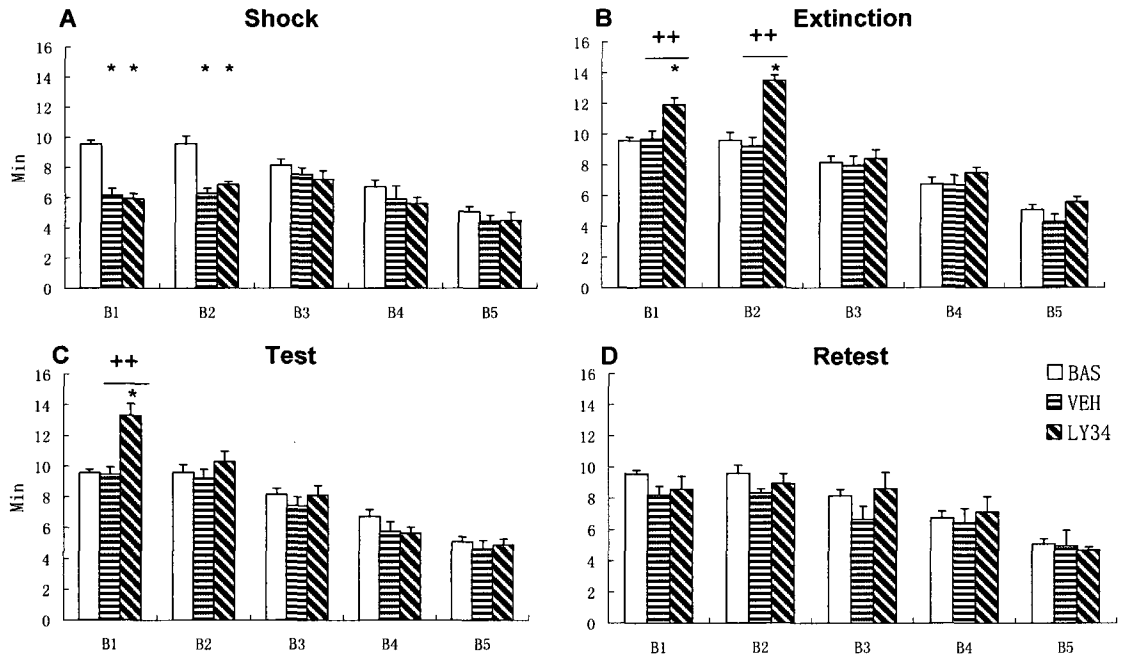


Fig. 17 - Time spent in REM plotted in 4 h blocks for 20 h after Shock training (A), Extinction training (B), Testing (C) and Retesting (D). * $p<0.05$, significant differences relative to the baseline (BAS); ++ $p<0.05$, significant differences between the VEH group and the LY34 group. Comparisons were conducted using Tukey tests.

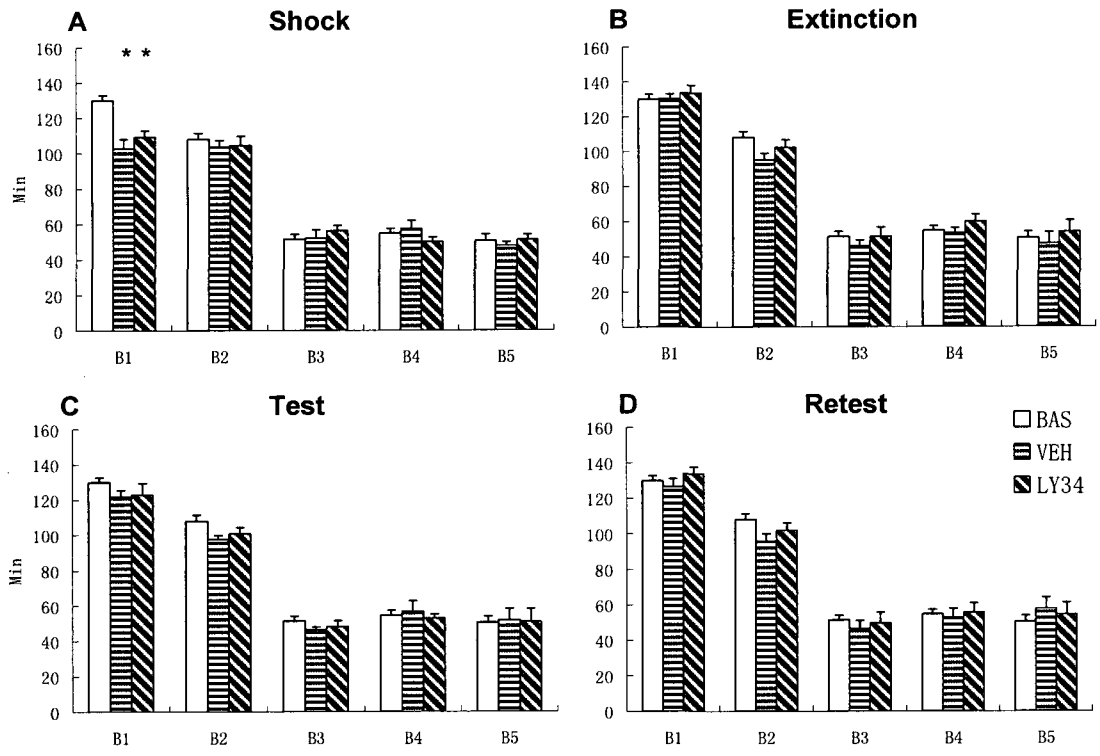


Fig. 18 - Time spent in NREM plotted in 4 h blocks for 20 h after Shock training (A), Extinction training (B), Testing (C) and Retesting (D). * $p<0.05$, significant differences relative to the baseline (BAS). Comparisons were conducted using Tukey tests.

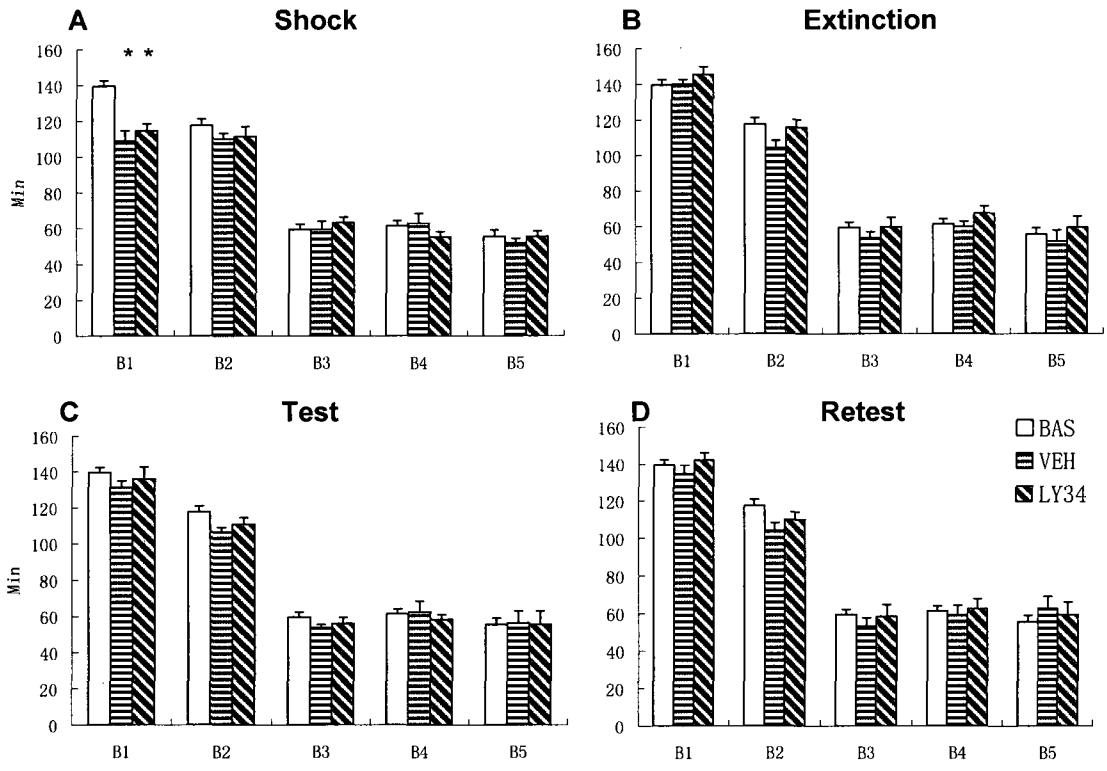


Fig. 19 - Time spent in Total Sleep plotted in 4 h blocks for 20 h after Shock training (A), Extinction training (B), Testing (C) and Retesting (D). * $p<0.05$, significant differences relative to the baseline (BAS). Comparisons were conducted using Tukey tests.

Table 3 - REM Episode Numbers and REM Average Durations (mean±SEM) during 4 h blocks following each treatment.

Conditions	Groups	B1	B2	B3	B4	B5
REM Episode Number						
Baseline	Combined	9.1 (0.2)	9.5 (0.3)	8.0 (0.2)	8.6 (0.3)	5.7 (0.3)
Shock	VEH	5.0 (0.3)***	5.7 (0.2)***	7.6 (0.4)	8.3 (0.5)	4.9 (0.3)
Shock	LY34	4.8 (0.4)***	5.9 (0.5)***	7.8 (0.5)	8.8 (0.4)	4.9 (0.5)
Extinction	VEH	9.0 (0.3)	9.4 (0.2)	7.9 (0.3)	8.4 (0.3)	5.3 (0.3)
Extinction	LY34	10.1 (0.3)	11.6 (0.5)***	8.4 (0.3)	8.4 (0.4)	5.9 (0.4)
Test	VEH	9.0 (0.2)	8.7 (0.2)	7.7 (0.2)	8.4 (0.2)	5.0 (0.4)
Test	LY34	11.6 (0.3)***	9.0 (0.3)	7.5 (0.2)	8.1 (0.4)	5.8 (0.3)
Retest	VEH	8.4 (0.3)	8.7 (0.3)	7.6 (0.5)	8.1 (0.3)	5.6 (0.4)
Retest	LY34	8.6 (0.5)	9.1 (0.4)	8.3 (0.3)	8.0 (0.6)	5.0 (0.6)
REM Average Duration						
Baseline	Combined	65.7 (1.9)	68.0 (1.5)	60.4 (2.2)	50.9 (2.3)	44.4 (1.2)
Shock	VEH	55.7 (2.4)***	58.4 (2.2)**	56.1 (1.3)	53.6 (2.5)	43.4 (2.8)
Shock	LY34	51.2 (2.9)**	58.4 (3.1)**	56.6 (3.1)	49.5 (2.5)	40.2 (1.5)
Extinction	VEH	65.7 (3.3)	62.8 (3.2)	60.4 (4.2)	51.1 (3.1)	46.6 (2.6)
Extinction	LY34	63.9 (2.7)	68.7 (5.4)	59.5 (7.1)	54.4 (4.2)	44.1 (4.0)
Test	VEH	64.6 (4.9)	62.0 (2.9)	57.0 (2.8)	49.5 (7.0)	48.4 (2.3)
Test	LY34	65.5 (2.8)	65.0 (4.3)	53.6 (3.6)	55.4 (2.5)	47.6 (5.0)
Retest	VEH	59.6 (6.0)	70.3 (4.6)	57.1 (2.7)	46.9 (3.2)	37.6 (8.0)
Retest	LY34	65.6 (8.1)	64.2 (2.8)	58.7 (3.3)	53.1 (4.8)	41.5 (2.5)

* $p<0.05$, ** $p<0.01$, *** $p<0.001$; significant differences compared to the baseline. Comparisons were conducted using Tukey tests.

Sleep on the Extinction Training Day

The ANOVA for total REM amounts across treatments and time blocks found a significant main effect for the treatment [$F(2, 4)=16.882$, $p<0.001$] and a significant interaction of treatment X time block [$F(4, 8)=3.041$, $p<0.01$]. Compared to the baseline, there was no significant difference for the VEH group across the first and second 4 h blocks (Fig. 17B). In contrast, extinction significantly increased REM in the LY34 group compared to the VEH group in the first ($p<0.05$) and second 4 h block ($p<0.001$) as well as compared to the baseline in the first 4 h block ($p<0.01$) and second 4 h block ($p<0.001$).

The ANOVA for total NREM amounts found no significant effect for the treatment [$F(2, 4)=2.460$, $p=0.089$]. The VEH group and the LY34 group showed no significant difference in NREM (Fig. 18B) in any of the five 4 h blocks. However, the ANOVA for total sleep amounts found a significant effect for the treatment [$F(2, 4)=3.468$, $p<0.05$]. There was an overall increased total sleep for the LY34 group compared to the VEH group ($p<0.05$).

The ANOVA for REM episode numbers during the extinction training day found a significant main effect for the treatment [$F(2, 4)=8.939$, $p<0.001$] and a significant interaction of treatment X time block [$F(4, 8)=2.533$, $p<0.05$]. Extinction training significantly increased the REM episode number in the LY34 group during the second 4 h block compared to both the baseline and the VEH group (Table 3, $p<0.001$). The ANOVA for REM average durations did not find any significant difference.

Sleep on the Test Day

The ANOVA for total REM amounts across treatments and time blocks found a

significant main effect for treatment [$F(2, 4)=4.345$, $p<0.05$] and a significant interaction of treatment X time block [$F(4, 8)=3.285$, $p<0.01$]. Compared to the baseline, there was no significant difference for the VEH group during the first and second 4 h blocks (Fig. 17C). However, there was significantly increased REM in the LY34 group compared to the VEH group in the first 4 h block ($p<0.001$) as well as compared to the baseline in the same period ($p<0.001$).

The ANOVA for total NREM amounts found no significant effect for the treatment [$F(2, 4)=2.807$, $p=0.064$]. Similarly the ANOVA for total sleep amounts found no significant effect for the treatment [$F(2, 4)=2.365$, $p=0.098$].

The ANOVA for REM episode numbers during the test day found a significant main effect for the treatment [$F(2, 4)=4.388$, $p<0.05$] and a significant interaction of treatment X time block [$F(4, 8)=5.818$, $p<0.001$]. The REM episode number was significantly increased in the LY34 group during the first 4 h blocks compared to both the baseline and the VEH group (Table 3, $p<0.001$). The ANOVA for REM average durations did not find any significant difference.

Sleep on the Retest Day

The ANOVA for total REM amounts across treatments and time blocks found no significant effect for the treatment [$F(2, 4)=2.425$, $p=0.092$]. The ANOVA for total NREM amounts found no significant effect for the treatment [$F(2, 4)=0.648$, $p=0.525$]. Similarly the ANOVA for total sleep amounts found no significant effect for the treatment [$F(2, 4)=0.974$, $p=0.380$].

The ANOVA for REM episode numbers or average durations did not find any significant differences.

2.4. Sleep Comparisons across Training Conditions among Each Group

We compared select sleep parameters in the light period across conditions separately in the VEH group and the LY34 group. Fig. 20 presents average REM (Fig. 20A, D), NREM (Fig. 20B, E) and total sleep (Fig. 20C, F) amounts in two 4 h blocks for the VEH and the LY34 group, respectively.

Sleep Comparisons across Training Conditions in the VEH Group

The ANOVA for total REM amounts across training found a significant effect for training [$F(4, 24)=9.302$, $p<0.001$]. Compared to the baseline, shock training significantly suppressed REM in both 4 h blocks ($p<0.01$) whereas extinction training significantly increased REM compared to shock in both 4 h blocks ($p<0.01$) and normalized REM to the baseline level. On the test day, animals in the VEH group showed higher REM in the first ($p<0.001$) and second ($p<0.01$) 4 h block compared to equivalent time periods on the shock training day. During the retest day, REM was also significantly higher in both 4 h blocks ($p<0.05$) than on the shock training day.

The ANOVA for total NREM amounts across training found a significant effect for training [$F(4, 24)=7.943$, $p<0.001$]. Shock training significantly suppressed NREM in the first 4 h block ($p<0.01$), whereas NREM increased following extinction ($p<0.001$), testing ($p<0.05$) and retesting ($p<0.01$) during the same period. The ANOVA for total sleep amounts across training found a significant effect for training [$F(4, 24)=8.594$, $p<0.001$]. Compared to the baseline, shock training significantly suppressed total sleep in the first 4 h block ($p<0.01$). By comparison, total sleep was increased following extinction ($p<0.001$), test ($p<0.01$) and retest ($p<0.01$) sessions during the first 4 h block compared to that period after shock training.

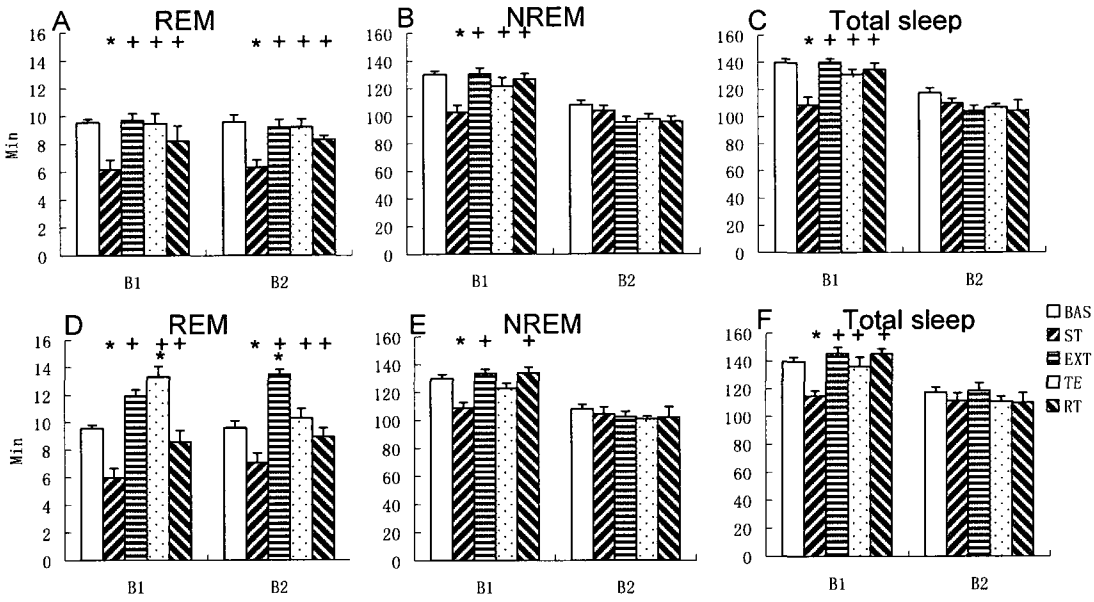


Fig. 20 - Average REM (A, D), NREM (B, E) and Total Sleep (C, F) plotted in two 4 h blocks in the VEH group (A, B and C) and the LY34 group (D, E and F), respectively. * $p<0.05$, significant differences relative to the baseline (BAS); + $p<0.05$, significant differences relative to shock period (ST). EXT: extinction training; TE: testing; RT: retesting. Comparisons were conducted using Tukey tests.

Sleep Comparisons across Training Conditions in the LY34 Group

The ANOVA for total REM amounts across training found a significant effect for training [$F(4, 28)=26.455$, $p<.001$]. Compared to the baseline, shock training significantly suppressed REM in first 4 h block ($p<0.001$) and second 4 h block ($p<0.05$). Extinction training significantly increased REM when compared to shock during both 4 h blocks ($p<0.001$) and when compared to the baseline in the second 4 h block ($p<0.001$). Following the test session, animals showed higher REM in the first ($p<0.001$) and second ($p<0.001$) 4 h block compared to those periods during the shock training day. Also, the animals showed increased REM compared to the baseline in the first 4 h block ($p<0.01$). On the retest day, REM was significantly higher in both 4 h blocks ($p<0.05$) compared to those periods on the shock training day.

The ANOVA for total NREM amounts found a significant effect for training [$F(4, 28)=7.127$, $p<0.001$]. Shock training significantly suppressed NREM in the first 4 h block ($p<0.01$), whereas NREM was increased following extinction ($p<0.01$) and retest ($p<0.01$) during the same period.

The ANOVA for total sleep amounts also found a significant effect for training [$F(4, 28)=9.317$, $p<0.001$]. Shock training significantly suppressed total sleep in the first 4 h block ($p<0.001$), whereas total sleep was increased during the first 4 h block following extinction ($p<0.001$), test ($p<0.01$) and retest ($p<0.01$) sessions compared to the same period.

3. Discussion

The current results demonstrate that mGlu II receptors in the BA may play a role in regulating fear extinction. Microinjection of the mGlu II antagonist, LY34, exacerbated

fear responses induced by contextual environment in the early period (P1) of extinction training. However, rats receiving LY34 showed attenuated fear responses in the later periods (P2, P3 and P4), suggesting that mGlu II receptors may exert a regulatory role in fear responses. Also, we observed a significant increase in REM in the LY34 group following both extinction training and on the test day compared to the VEH group. However, we did not observe any significant difference in NREM or total sleep between the LY34 and VEH groups. These data suggest that the BA may also be involved in the regulation of REM.

The level of freezing has been used as an index for measuring fear, with higher FT% indicating greater fear reactions (Blanchard and Blanchard, 1969; Doyere et al., 2000). In rodents, shock training can significantly increase FT% to a relatively high level during shock and post-shock periods compared to that of the pre-shock period, indicating that animals successfully acquired conditioned fear (Tang et al., 2005; Wellman et al., 2008). Contextual fear can induce an increased FT% level similar to shock. However, the level of FT% is typically reduced after animals are repeatedly exposed to the context without shock. It has been reported that rats exhibited a cessation of freezing by the end of a one-hour long session, which indicated that fear extinction occurred (Wellman et al., 2008). In the current study, both groups in early periods of extinction (P1 and P2) exhibited higher FT% compared to later periods (P3 and P4). These data are consistent with our previous finding that the context can induce fear responses, while extinction training can reduce fear responses. Interestingly, the microinjection of LY34 in the BA exacerbated fear responses in P1 and reduced fear responses in the later periods (P2, P3 and P4) when compared to the VEH group. Also the LY34 group exhibited lower FT%

compared to the VEH group during the test and retest. These data suggest that LY34 facilitates fear extinction that reduces fear responses.

It has been reported that mGlu II receptors are implicated in cognitive performance. For example, systemic injection of the agonist LY35 induces a cognitive impairment in which rats demonstrates a delay-dependent deficit in the delay-match or non-match task and impaired spatial learning in the Morris water maze. In contrast, systemic injection of LY34 can block the effects of LY35 in the delay-match or non-match task and increase spatial learning in the Morris water maze (Higgins et al., 2004; Shimazaki et al., 2007). A possible mechanism may be that administration of LY35 inhibits neuronal transmission in the perforant path inputs to both the dentate gyrus and the stratum lacunosum moleculare of the CA1 in the hippocampus (Kew et al., 2001). Evidence indicates that the amygdala can modulate cognition and learning through mediating stress effects on hippocampal long-term potentiation (LTP) (Kim et al., 2001). For instance, blockage of the BLA impairs spatial learning (Roozendaal et al., 1998); lesion of the CNA impairs attention during Pavlovian fear conditioning (Holland et al., 2000); and lesion of the BLA impairs *in vivo* LTP in the dentate gyrus of the hippocampus (Ikegaya et al., 1994). In addition, the amygdala sends projections to the hippocampus (Sah et al., 2003), through which it may potentially modulate hippocampal function. Therefore, we propose that microinjection of LY34 into the BA may modulate the activity of the amygdala and enhance cognitive performance and learning through the hippocampus. The enhancement of an animal's recognition of the contextual environment may trigger a higher fear response in the beginning of fear extinction, and then later facilitate fear extinction.

The alteration of REM has often been associated with stress and fear (Sanford et al.,

1995; Sanford et al., 2001; Jha et al., 2005; Wellman et al., 2008). Previous work in our lab has found that footshock induces suppression of REM without later recovery and fearful contexts can induce similar effects on REM in both rats and mice (Sanford et al., 2003; Tang et al., 2005). In the current study, we found that shock training reduced REM in both the VEH and LY34 groups. By comparison, extinction training on the following day significantly increased REM. Following the test on the third day, the LY34 group again demonstrated significantly increased REM compared to the VEH group. Considering that administration of LY34 in the BA alone suppressed REM, extinction learning may be the reason for the increased REM in the LY34 group. These data suggest that injection of LY34 may facilitate the effects of extinction training, which in turn increases REM.

Shock suppressed NREM in both groups while extinction training was found to restore it to the baseline level. We observed similar results for total sleep. These data are consistent with results showing that extinction training significantly increases NREM and total sleep compared to that following shock training one day before (Wellman et al., 2008).

Fear extinction has been studied for decades and is currently considered to be a learning process that forms new circuits that inhibit old conditioned reactions (Quirk and Mueller, 2008). Failure of extinction of conditioned fear may be the mechanism underlying persistent anxiety reactions, such as posttraumatic stress disorder (PTSD) (Pitman, 1997). Various evidence indicates that the mPFC and the amygdala are involved in fear extinction and it has been proposed that the mPFC inhibits conditioned fear through inhibition of CNA outputs (Quirk et al., 2003). Anatomically, the BA appears to

be located at a potentially crucial position for contextual fear extinction as it has reciprocal connections with the mPFC, the hippocampus and the CNA (Sah et al., 2003). Inhibition of NMDA receptors in the BA disrupts the expression and extinction of conditioned fear to contextual stimuli (Lee and Kim, 1998). These data suggest that the BA may be an essential site for fear conditioning and extinction. In the current study, LY34 in the BA facilitated fear extinction as indicated by changes in FT% and REM. These results support for a role of the BA in the regulation of fear extinction. These results also demonstrate for the first time that mGlu II receptors may affect fear extinction.

Injection of LY34 enhances neuronal activity, as indicated by the increase of c-Fos expression in the brain (Linden et al., 2005; Hetzenauer et al., 2008). The BA projects to prelimbic (PL) neurons of the mPFC, which in turn project to the infralimbic (IL) neurons of the mPFC. The IL sends extensive excitatory projections to the LA and the intercalated cell masses (ITC). In contrast, the PL sends projections back to the BA (McDonald et al., 1996). This circuitry may be involved in the observed suppression of freezing. Extinction learning stimulates pyramidal cells in the BA, which send out excitatory outputs to the PL. The PL stimulates the IL which sends excitatory signals to the ITC, which, in turn, inhibits the CNA. The inhibition of the CNA may regulate the suppression of freezing (Sotres-Bayon et al., 2004). Thus, the injection of LY34 may increase neuronal activity in the BA, which sends out stronger excitatory stimulus to the PL and the IL, thus producing stronger inhibition in the CNA. This could account for the lower freezing level in the LY34 group compared to the VEH group. This conception is consistent with current literature explaining fear extinction. However, it does not explain

the enhancement of REM, which likely involves increased activation of the CNA (Sanford et al., 2002; Sanford et al., 2006). Therefore, additional work is needed to reconcile the mechanisms that underlie the increases of REM with those that regulate fear memory, as indicated by freezing.

Conditioned fear has been linked to the development of anxiety disorders including PTSD (Shalev et al., 1992). The failure of fear extinction appears to be an important factor in PTSD (Peri et al., 2000) and extinction-based exposure therapies are widely used for its treatment as well as other anxiety disorders (Foa, 2006). Sleep disturbances, including significant alterations in REM, are common features of PTSD (Germain et al., 2008; Sateia, 2009). However, there has been virtually no research on the neural processes that may underlie the relationship between fear extinction and sleep. This study is the first research to explore the role of mGlu II receptors in extinction learning and subsequent sleep.

Also in this study, we examined the spontaneous recovery of fear following extinction (Fig. 20). In the short-term (test day, one day following extinction day) and long-term (retest day, nine days following extinction day), we did not find any spontaneous recovery of fear in either group as evidenced by REM, a sensitive index for fear (Jha et al., 2005). These data suggest that extinction training could ameliorate conditioned fear, which may provide approaches for the treatment of anxiety disorders, such as PTSD.

In summary, the role of the BA in regulating stress and stress-induced alterations in sleep has been minimally studied. Our data demonstrate that the BA may exert a role in fear-induced alterations in sleep. LY34 applied in the BA can facilitate fear extinction,

which in turn induces an increase of REM and NREM. The mechanism and pathways by which these effects are mediated require further study.

4. Experimental Procedure

4.1. Subjects

The subjects were fifteen male Wistar rats from Harlan (Indianapolis, IN) of approximately 300gm at the time of surgery. The rats were individually housed in polycarbonate cages and had ad lib access to food and water. The room for sleep recording/housing was kept on a 12:12 light:dark cycle with lights on from 7:00 AM to 19:00PM. The room temperature was maintained at 24.5°C ± 0.5°C.

4.2. Surgery

One week following their arrival, the rats were implanted with skull screw electrodes placed in the skull for recording the electroencephalogram (EEG) and with stainless steel wire electrodes sutured into the nuchal muscle for recording the electromyogram (EMG). Leads from the implanted electrodes were led subcutaneously to the skull and routed to a 9-pin miniature plug attached to the skull. Guide cannulae (26 gauge) for microinjections were bilaterally implanted to introduce the tip of the injection cannulae into the target areas at the BA (AP=-2.6, DV=8.0, ML= ±4.8 mm (Paxinos G, 1997)). All surgeries were conducted under aseptic conditions. The rats were anesthetized with isoflurane (5% induction; 2% maintenance). In addition, ibuprofen (15 mg/kg) was available in water 24 h before surgery and 3 days after surgery to alleviate pain. All procedures were conducted in accordance with the National Institutes of Health *Guide for the Care and Use of Experimental Animals* and were approved by Eastern Virginia Medical School's Animal Care and Use Committee (Protocol #07-005).

4.3. Drug preparation and Microinjection Procedure:

The mGlu II receptor antagonist, LY34, was purchased from Tocris Bioscience (Ellisville, MO). Immediately prior to the microinjection, LY34 was dissolved in 0.01M phosphate buffered saline (PBS). PBS alone was microinjected into the vehicle control animals.

Microinjections were administrated immediately before the start of extinction training. Eight rats in the experimental group received microinjection of antagonist LY34 (30 nM, 0.5 μl) while seven rats in the control group received microinjection of vehicle PBS (0.01 M, 0.5 μl).

For microinjections, injection cannulae (33 ga.), which projected 1.0 mm beyond the tip of the guide cannulae, were secured in place within the guide cannulae. The injection cannulae were connected to lengths of polyethylene tubing that in turn were connected to 5.0 μl Hamilton syringes. The injection cannulae and tubing had been pre-filled with the solution to be injected. The solutions in a volume of 0.5 μl were slowly infused over 3 min, with the cannulae left in place 1 min prior to the start and 1min after the completion of the microinjection.

4.4. Experimental Procedures

After surgery, the rats were allowed 14 days for recovery. Then they were habituated to microinjection procedures for two days. After each habituation handling, the rats were connected to cables for 20 h sleep recording.

After habituation, the rats were randomly assigned to one of the following two groups: the experimental group in which rats would be injected with LY34 (30 nM, 0.5 μl) immediately before extinction training and the control group in which rats would be

injected with vehicle (0.01 M PBS, 0.5 μl) immediately before extinction training.

Baseline sleep was recorded for 20 hours. Two days after baseline recording, the rats received shock-training. Individual rats were placed in shock chambers (Coulbourn Habitest cages equipped with grid floors (Model E10-18RF) that were housed in Coulbourn Isolation Cubicles (Model H10-23)). The rats were allowed to freely explore the shock chamber for 5 minutes, after which they were presented with 20 footshocks (0.8 mA, 0.5-second duration) at 1.0-minute intervals over the course of 20 minutes. Five minutes after the last shock, the rats were returned to their home cages and connected to the cables for sleep recording.

The day following shock training, the rats were microinjected into the BA with either LY34 or vehicle. Then they were returned to the shock chambers and allowed to freely exploring the chamber for 1 hour for extinction training. The next day the rats were put in the same chamber for the test of extinction effects (test day). Eight days following the test day, rats were returned to the same chamber for the test of their recall of fear for the context (retest day). The shock chambers were thoroughly cleaned with diluted alcohol following each training or test session.

Each session was videotaped using mini video cameras (Weldex, WDH-2500BS, 3.6mm-lens) attached to the center ceiling of the shock chamber.

All experimental procedures were conducted in the fourth hour of the light period, so the sleep recording started from the beginning of the fifth hour of light period. Sleep was recorded for 20 hour following each training/test session.

4.5. Analysis of Freezing

Freezing behavior, defined as the absence of body movement except for respiration,

was visually determined from the videotapes by a trained observer. Freezing was scored in 5-second intervals during each observation time block periods over the course of the recording period when the rats were placed in the shock chamber. During shock training, the preshock and postshock periods were scored for freezing (the first and last 5 minutes of the recording). During shock presentation, freezing was scored following shock presentations. For test and retest of contextual fear, freezing was scored over 30 minutes. For the extinction training, freezing was scored for four 15 minutes periods. The freezing time percentage (freezing time/total time × 100%) was calculated for each animal.

4.6. Sleep Recording

Sleep recording was performed with rats in their homecages placed in a chamber equipped for electrophysiological recording. A lightweight, shielded cable was connected to the plug in the rats' head. The cable was connected to a swivel that allowed free movement for the rats within their cages. EEG and EMG signals were processed by a Grass Model 12 polygraph equipped with model 12A5 amplifier and routed to an A/D board (Eagle PC30) housed in a Pentium class PC. The signal were digitized at 128 HZ and collected in 10-second epochs using a custom sleep data collection program.

4.7. Determination of Sleep

The EEG and EMG records were visually scored by a trained observer to determine wakefulness, NREM and REM in 10-second epochs (Sanford et al., 2002). Wakefulness was determined by the presence of low-voltage, fast EEG; high-amplitude, tonic EMG levels; and phasic EMG bursts that could be associated with gross body movements. NREM was scored by the presence of spindles interspersed with slow waves, lower muscle tone, and no gross body movements. REM was determined by the presence

of low-voltage, fast EEG, theta rhythm, and muscle atonia.

4.8. Data Analysis

Statistical analyses were conducted using SigmaStat (SPSS, Inc., Chicago, Illinois). Two-way ANOVAs across drug treatment and 4 h blocks were used to analyze freezing behavior. For comparison of sleep data between groups in each training day, one way ANOVAs were used for each 4 h block. For comparison of sleep data across training day for each group, one way repetitive ANOVAs were used for each 4 h block.

CHAPTER IV

SUMMARY

The amygdala has been found to be implicated in the emotional modulation of sleep (Chapter I). The BA is a specific amygdaloid area that receives sensory inputs and projects to the CNA and the BNST, which have direct connections with hypothalamic and brainstem areas involved in the regulation of sleep and stress. In this study, we propose that the BA is an essential brain area involved in the regulation of normal sleep and in stress-induced alterations in sleep, and that mGlu II receptors could play a direct role in such regulation. Therefore, as discussed in chapter II, the specific mGlu II receptor agonist LY37 or antagonist LY34 was microinjected into the BA.

LY35 may be safely microinjected into the amygdala at millimolar concentrations (Walker et al., 2002). Since LY37 is a derivative of LY35 and has similar characteristics, we assumed that LY37 could be safely injected into the BA at millimolar levels. We found that injection of LY37 suppressed REM without affecting NREM and wakefulness. However, we did not observe any dosage response. It has been reported that the affinity of LY37 for mGlu II receptors can be determined by evaluating its ability to displace radio-labeled antagonist LY34 (Monn et al., 1999). LY37 binds mGlu II receptors with a high affinity (IC50=10 nM) to mGlu II receptors in rat brain homogenates (Imre, 2007). Since mGlu II receptors are negatively coupled to adenylate cyclases, the ability of LY37 to suppress forskolin-stimulated cAMP production is another important parameter for the pharmacological characteristics of LY37 (Monn et al., 1999). It has been reported that LY37 suppresses cAMP production in cells expressing human mGlu2 receptors (EC50=3

nM) and mGlu3 receptors (EC50=6 nM) (Imre, 2007). These data suggest that LY37 has a high affinity for mGlu II receptors. Therefore, we reduced drug concentrations to nanomolar levels and observed a dose-dependent response. This result is consistent with the high affinity of mGlu II receptors ligands. Therefore, for the antagonist experiments, we injected LY34 at nanomolar levels and observed increased arousal accompanied by suppression of REM and NREM in a dose-dependent manner. These data support the conclusion that the BA is an important brain area for the regulation of sleep by mGlu II receptors.

The BA is also implicated in conditioned fear and fear extinction (Davis and Whalen, 2001; Sotres-Bayon et al., 2004). Therefore, as discussed in chapter III, animals received a microinjection of LY34 (30 nM) or vehicle alone into the BA prior to extinction training and subsequent recording of their behavior and sleep. It was found that with the injection of LY34, the animals demonstrated less fear responses after extinction training compared to the control group. It was also found that the LY34 treated animals had increased REM following extinction training and testing. Since the microinjection of LY34 alone suppressed REM, extinction training may be the factor that induced the increase of REM. These data confirmed that fear and fear extinction could have an impact on the subsequent sleep, especially REM.

Therefore, we conclude that the BA plays a role in the regulation of sleep and that mGlu II receptors in the BA are important in that regulation. Moreover, the BA is also an area involved in conditioned fear and fear extinction. Manipulation of the BA by mGlu II receptor agents could have a direct impact on extinction effects and subsequent sleep. Although the exact mechanisms are still unclear, a generally accepted hypothesis is that

mGlu II receptors serve as autoreceptors and function primarily during high glutamate release. We propose that injection of LY37 produces no significant alteration in glutamate release but suppresses GABA release (Anwyl, 1999; Cartmell and Schoepp, 2000; Smolders et al., 2004). It is the decrease in GABA associated with specific neuronal circuitry in the BA, the ITC and the CNA that contributes to the suppression of REM. Moreover, mGlu II receptors may maintain a tonic activity for modulating glutamate release (Linden et al., 2005). Suppression of mGlu II receptors by LY34 may shift glutamate release to a higher level and thus increase neuronal activity in the BA. This may be the reason that injection of LY34 in the BA increased arousal and suppressed REM and NREM. This increased neuronal activity in the BA may also contribute to the enhanced learning of extinction training, which may be responsible for the increased REM during the extinction day and testing day.

Altogether, this study for the first time identified the BA as a potential brain area through which mGlu II receptors could regulate sleep. The combination of the mGlu II antagonist LY34 with extinction training ameliorated fear responses and sleep disturbances following conditioned fear. This may provide new prospects for the development of treatments for anxiety disorders, such as PTSD.

Through the current studies we found that mGlu II receptors are involved in the regulation of sleep and stress-induced alterations in sleep. We proposed two separate pathways as indicated in Fig. 21 and Fig. 22 to explore the possible underlying mechanism. As illustrated in Fig. 21, microinjection of LY37 in the BA suppresses GABA release, which in turn suppresses the interneuron activity. The pyramidal neurons in the BA receive decreased inhibitory inputs from interneurons and send increased

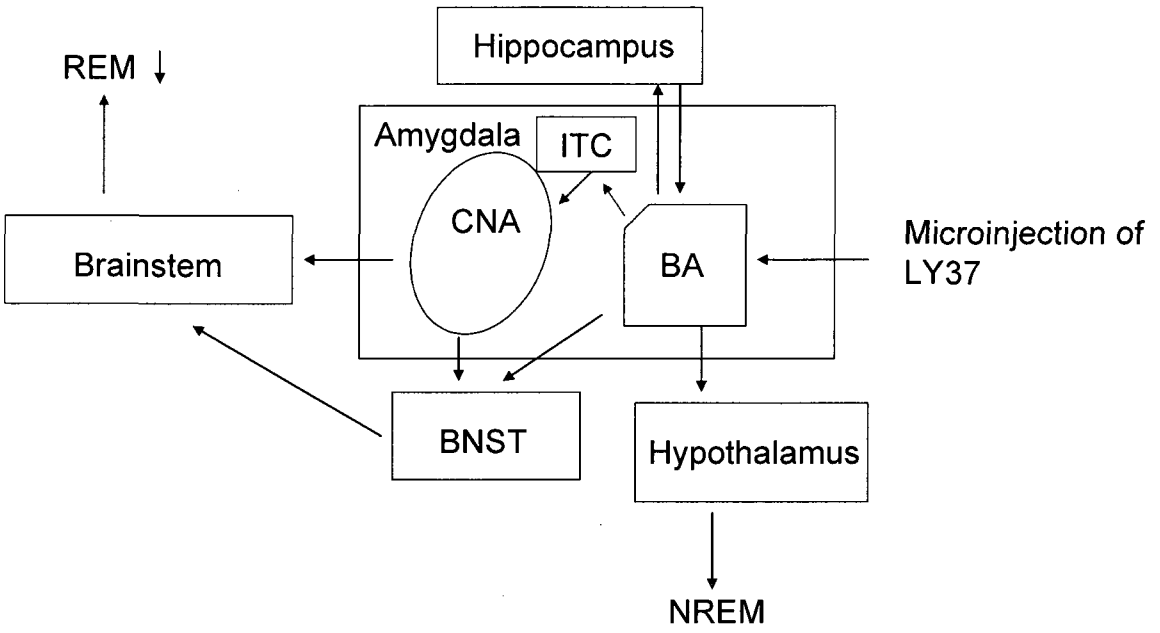


Fig. 21 - Proposed pathway by which microinjection of LY37 regulates REM through the BA. Microinjection of LY37 suppresses GABA release in the BA, which suppresses the activity of interneurons in the BA. The pyramidal neurons in the BA receive decreased inhibitory inputs from interneurons and send increased excitatory signals to intercalated cells. Therefore the BA sends increased excitatory outputs to intercalated cells. Intercalated cells in turn send increased inhibitory signals to the CNA and suppress REM. ITC: intercalated cells.

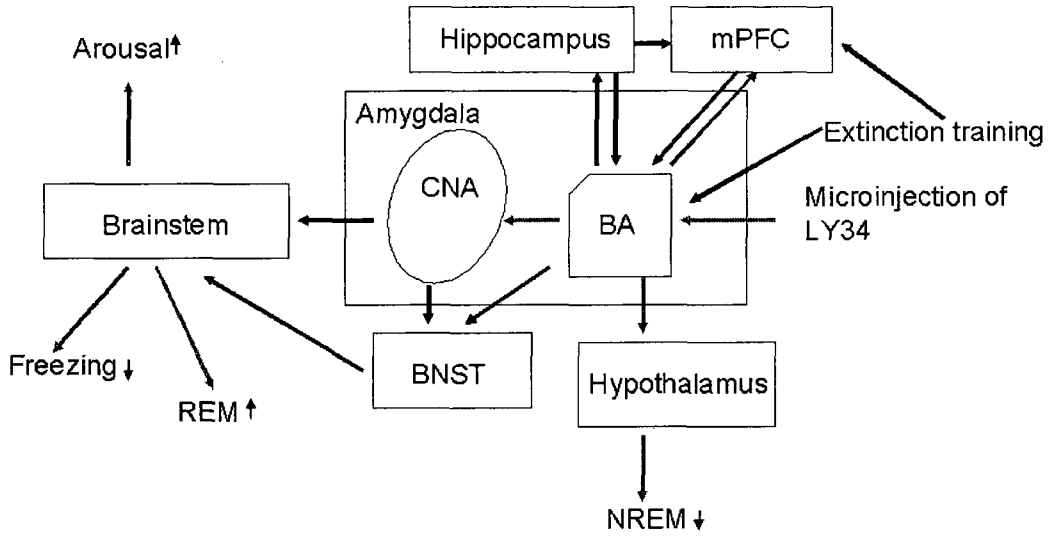


Fig. 22 - Proposed pathway by which microinjection of LY34 and extinction training regulate fear responses and sleep. Microinjection of LY34 increases neuronal activity in the BA, which suppresses REM and increase arousal through the CNA pathway as well as suppresses NREM through the hypothalamus. The increased neuronal activity in the BA may also facilitate fear extinction learning which may be responsible for the enhanced REM following extinction training and testing. ITC: intercalated cells.

excitatory signals to intercalated cells. Therefore the BA sends increased excitatory outputs to intercalated cells. Intercalated cells in turn send increased inhibitory signals to the CNA and suppress REM. As illustrated in Fig. 22, microinjection of LY34 increases neuronal activity in the BA, which suppresses REM and increases arousal through a CNA pathway as well as suppresses NREM through the hypothalamus. The increased neuronal activity in the BA may also facilitate fear extinction learning which may be responsible for the enhanced REM following extinction training and testing. These pathways are presented according to currently available evidence in the literature. Therefore, for future directions, we would like to expand the investigation by exploring the underlying mechanism of the behavioral observations. We would like to analyze the cell activity level in the BA after injection of LY37 by using c-Fos staining. The cells would be grouped into three categories: pyramidal cells, interneurons and intercalated neurons. The c-Fos expression level would be analyzed in these three types of cells to determine any change compared to the baseline. We would also like to perform electrophysiological recording of specific cell types prior to or after injection of LY37 or LY34 to determine the effects of the drug.

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Publications:

1. Sanford LD, Yang L, Wellman LL, **Dong E**, Tang X (2008). Mouse strain differences in the effects of corticotropin releasing hormone (CRH) on sleep and wakefulness. Brain Res 1190:94-104.

2. Sanford LD, Yang L, Tang X, **Dong E**, Ross RJ, Morrison AR (2006). Cholinergic regulation of the central nucleus of the amygdala in rats: effects of local microinjections of cholinomimetics and cholinergic antagonists on arousal and sleep. Neuroscience 141(4):2167-2176.

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