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# Comparative analysis of the neurophysiological profile of group II metabotropic glutamate receptor activators and diazepam: Effects on hippocampal and cortical EEG patterns in rats

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ABSTRACT

Selective activation of the Group II metabotropic glutamate receptors 2/3 (mGlu2/3) by either full agonists or positive allosteric modulators (PAMs) show anxiolytic activity. In the present study the anxiolytic profile of mGlu2/3 receptor agonists LY-354740 and LY-404039 and the mGlu2 receptor PAM 1-methyl-2-((cis-3-methyl-4-(4-trifluoromethyl-2-methoxy)-phenyl)piperidin-1-yl)-1H-imidazo[4,5-b] pyridine (MTFIP) were evaluated using neurophysiology-based assays. Activation of mGlu2/3 receptors by these compounds, as well as the positive control diazepam, significantly decreased the frequency of hippocampal theta oscillation elicited by stimulation of the brainstem nucleus pontis oralis (nPO), a characteristic action of anxiolytic compounds. Since the nPO is a critical region involved in regulation of rapid eye movement sleep, mGlu2/3 receptor activators were also tested on sleep parameters, as well as on cortical and hippocampal encephalography (EEG) activity. Both mGlu2/3 agonists and the mGlu2 PAM significantly prolonged REM sleep latency and reduced total REM sleep duration while during the active awake state all compounds lowered hippocampal peak theta frequency. However, diazepam and mGlu2/3 agonists/PAM elicited opposite changes in cortical EEG delta and beta bands. Delta power significantly increased after any of the mGlu2/3 compounds but decreased after diazepam. In the beta band, mGlu2/3 receptor agonists dose-dependently decreased beta power in contrast to the well-known beta activation by diazepam. These effects lasted 3–4 h and could not be explained by modest, transient changes (<1 h) in waking and slow wave sleep. The current observations support the role of mGlu2/3 receptor activators as potential anxiolytic compounds, but indicate a distinct action on cortical EEG activity which is different from the effects of $GABA_A$ PAMs.

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## 1. Introduction

The Class II metabotropic glutamate receptor subtypes mGlu2 and mGlu3 are G-protein coupled receptors located primarily on presynaptic terminals that when activated by the endogenous neurotransmitter glutamate, or by one of several known specific agonists or positive allosteric modulators (PAMs), act to decrease neuronal excitability by inhibiting further release of glutamate (Anwyl, 1999; Cartmell and Schoepp, 2000). Although mGlu2/3 receptors have been shown to be negatively coupled to the second messenger adenylate cyclase, the mechanism responsible for decreasing neurotransmitter release is thought to occur independently from cAMP regulation and at least in part through a membrane delimited inhibition of voltage gated, N-type $Ca^{2+}$ channels (Macek et al., 1996; Anwyl, 1999) although the cAMP cascade may play a regulatory role in this mechanism. Distribution of mGlu2/3 receptors in the brain is rather widespread being found in cortical and subcortical regions, including the olfactory bulb, entorhinal cortex, striatum, amygdala, thalamus, cerebellum and hippocampal formation (Wright et al., 2001). In the hippocampus, their expression is most intense within the lacunosum moleculare but can also be found in the molecular layers of the dentate gyrus, the hilus and stratum lucidum (Richards et al., 2005). This dense localization within such areas as the cortex and limbic regions, coupled with their regulatory role in glutamate-induced excitability, have thus prompted particular interest in these receptors as

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targets for psychiatric related drug therapies, particularly for anxiety and anxiety related disorders.

Indeed, there is mounting evidence that activation of mGlu2/3 receptors produces anxiolytic effects in both rodents as well as in humans. For example, in rodents, the mGlu2/3 receptor specific agonist LY-354740 has provided positive results in a variety of tests related to anxiety and/or stress such as fear-potentiated startle, the elevated-plus maze, lactate-induced panic, the Vogel drinking conflict test, stress-induced hypothermia and immobilization-induced stress (Helton et al., 1998; Tizzano et al., 2002; Walker et al., 2002; Galici et al., 2006; Johnson et al., 2005; Swanson et al., 2005; Lin et al., 2005; Rorick-Kehn et al., 2007). In humans, oral administration of LY-354740 has been shown to decrease $CO_2$-induced panic (Schoepp et al., 2003), attenuate fear-potentiated startle in healthy volunteers (Grillon et al., 2003) and decrease symptoms of generalized anxiety disorder (Dunayevich et al., 2008).

Recently, we have reviewed the neurophysiological effects of clinically active anxiolytic drugs on activity specifically related to hippocampal circuitry (McNaughton et al., 2007). In rats, high frequency (250 Hz) stimulation of the nucleus pontis oralis (nPO), a nucleus of the brainstem reticular formation, induces current-dependent theta oscillations in the hippocampus (Vertes, 1982) which can be modulated by the administration of anxiolytic drugs. Specifically, most pharmacologically active compounds shown to have utility as anxiolytics in humans, effectively reduce the frequency of stimulus-induced theta rhythm in the hippocampus of both anesthetized and non-anesthetized rats at doses that are clinically relevant (for review see McNaughton et al., 2007). Therefore, in the present study, the effects of the mGlu2/3 receptor agonists LY-354740 and LY-404039, as well as the mGlu2 positive allosteric modulator 1-methyl-2-((cis-3-methyl-4-(4-trifluoromethyl-2-methoxy)-phenyl)piperidin-1-yl)-1H-imidazo[4,5-b]pyridine (MTFIP, referred as (+)-17c in Zang et al., 2010) have been evaluated on stimulation-induced hippocampal theta activity in anesthetized rats using the clinically proven anxiolytic diazepam as a positive control.

It is also known that ascending neurons of nPO contribute to rapid eye movement (REM) sleep regulation (Xi et al., 2004) and previous studies have indicated that drug-related decreases in the frequency of hippocampal theta induced by nPO stimulation and reduction in REM sleep could share common mechanisms. For example, pregabalin reduces both the frequency of stimulation-induced theta oscillations as well as REM sleep in rats, most likely via attenuation of glutamate transmitter release (Siok et al., 2009; Kubota et al., 2001; Quintero et al., 2011). In terms of mGlu2/3 receptors, either direct activation, or positive allosteric modulation, have both been shown to prolong REM sleep latency and reduce total REM sleep in rodents (Feinberg et al., 2002; Ahnaou et al., 2009; Fell et al., 2011). Therefore, in addition to the stimulation induced hippocampal theta experiments performed in anesthetized animals, the present study also evaluates the effects of mGlu2/3 activation and diazepam on the sleep-waking activity of freely moving rats. In these studies, particular emphasis has been placed on both the hippocampal and cortical quantitative electroencephalography (EEG) measured during well defined behavioral states (wakefulness, REM sleep, behavioral exploration of novel environments) so as to assess 1) the effects of mGlu2/3 receptor activation on the relationship between changes in sleep-waking architecture in freely moving animals and brainstem stimulation-induced hippocampal EEG in anesthetized rats, 2) look for quantitative differentiation between activators of mGlu2/3 and diazepam and 3) provide a potential biomarker of mGlu2/3 receptor activation in the rat that is translatable to human studies.

## 2. Materials and methods

### 2.1. Anesthetized rat

#### 2.1.1. Animals and surgery

All procedures were carried out under an approved internal animal use protocol and in compliance with Animal Welfare Act Regulations (9 CFR parts 1, 2 and 3) and with the Guide for the Care and Use of Laboratory Animals, National Institutes of Health guidelines. Experiments were performed on male, Sprague Dawley rats (Harlan Laboratories, Indianapolis, IN) weighing 270-325 g and anesthetized with 1.5-1.6 g/kg IP urethane. Each animal was surgically implanted with an indwelling catheter (P50 tubing) into the left femoral vein for obtaining post-experiment blood samples and for administration of euthanizing doses of chloral hydrate. The catheter was flushed with 0.9% sterile saline and kept patent with 17 U/ml sodium heparin solution. Following cannulation, the animals were placed in a Kopf small animal model stereotaxic frame on a temperature regulated heating pad (Harvard Apparatus) set to maintain body temperature at 37-38 °C with the temperature monitored via rectal probe. The skull directly over the bregma-lambda suture was exposed followed by burring a small hole into the right frontal bone for placement of a stainless steel screw to act as the animal ground. Additional holes were burred into the left parietal bone to accept the recording electrode (hippocampal field CA1) and stimulating electrode (nucleus Pontis Oralis; nPO) according to the following coordinates (relative to bregma in mm; Paxinos and Watson, 1986): CA1, AP = −3.5, lateral = 2.0 and DV = −2.8 from the surface of the brain; nPO, AP = −7.6, lateral = 1.8 and DV = −6.0 from the surface of the brain. Following surgery, the animals were kept in the stereotaxic frame for the duration of the experiment and allowed to stabilize for 2-hours prior to beginning each recording. At the conclusion of each experiment, 0.5 cc of blood was drawn, followed by euthanization with a bolus injection of chloral hydrate, and the brains were removed for determination of electrode placement and for drug exposure analysis.

#### 2.1.2. Recording and stimulation

Field potentials (EEG; electroencephalogram) were recorded using bipolar, concentric stainless steel recording electrodes (NE-100X, Rhodes Medical Instruments, Woodland Hills, CA) and a Grass model P55 AC preamplifier with the filters set at 0.3–300 Hz and the 60 Hz notch filter in the off position. The signals were digitized at a rate of 1000 Hz (CED Micro 1401, Cambridge Electronic Design, Ltd, Cambridge UK) and stored off-line for subsequent analysis using Spike2 software (version 4, Cambridge Electronic Design). The brainstem was stimulated using the same type of bipolar concentric electrode used for recording the EEG and the stimulus was controlled using a Master8 stimulator coupled to an Iso-Flex stimulus isolator (A.M.P. Instruments, Ltd, Jarusalem Israel). Each stimulus consisted of a series of 0.3 ms square pulses delivered over a period of 6-second at a rate of 250 Hz. These 6-second trains were repeated for the duration of each experiment at an interval of every 100 seconds. However, the intensity of the anodal current varied in the following manner: The first train was delivered at 0.06 mA for the entire 6-second period. The second train, delivered 100 seconds after commencement of the first, was increased to 0.08 mA, and so on, increasing by 0.02 mA each time to a maximum of 0.16 mA by the sixth train in the series (i.e., 6 independent trains over a period of 600 seconds that varied from 0.06 to 0.16 mA). This pattern was repeated throughout the experiment thereby constructing a stimulus–response curve covering every sequential 10-minute time period. Following the surgical recovery period, each animal was given a series of stimulations (as outlined above) until a stable baseline could be established. Signals were considered stable if 3-consecutive 0.06–0.16 mA stimulation response (S–R) curves could be generated with little or no change in peak frequency (i.e. ±0.24 Hz) at each intensity. In addition, only animals whose peak frequency ranged from 5–6 Hz at the lowest stimulation intensity and 7–8 Hz at the highest were included in the study. Once the stability of the signal was established the experiment would begin by creating 3-consecutive S–R curves (i.e. 30 min) to establish the baseline response, followed by SC administration of either drug or vehicle ($n = 5$ each group). The EEG was then continuously monitored with a new 0.06–0.16 mA S–R being generated every 10 min. This procedure was carried out for 90 min post-dosing at the end of which plasma and brain tissue were collected as outlined above.

#### 2.1.3. Data analysis

Signals derived from the last 5-seconds of each stimulation period were quantified with Spike2 software by performing a 4096-point fast Fourier Transform (FFT) using a standard Hanning window which resulted in a frequency resolution of approximately 0.24 Hz. The first 1-second of each stimulation period was omitted to avoid the stimulus artifact. Peak theta frequency was computed by determining where the peak power occurred in the 4–9 Hz frequency band of the resultant power spectral plot. Total theta power was determined by summing the power computed over the same frequency range. For the stimulus–response plots of Fig. 1, peak theta frequency was averaged for each animal across each stimulation current during the 30 min period prior to drug injection, and the final 30 min period following drug injection (i.e. $n = 3$ S–R curves pre- and post-injection; five animals each per drug and dose). For the time-course analysis, average peak theta frequency was computed for each animal over every 0.06–0.16 mA stimulation set, then