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Inhibitory effects of jujuboside A on EEG and hippocampal glutamate in hyperactive rat*

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Abstract: In this study, the inhibitory effect of jujuboside A (JuA) on a penicillin sodium (Na-PCN) induced hyperactivity model was investigated. Cortical EEG (electroencephalogram) and the concentration of hippocampal Glutamate (Glu) were monitored simultaneously in vivo as indicators of rat's excitatory state. Power spectral density (PSD) and gravity frequency of PSD were calculated. JuA (0.05 g/L and 0.1 g/L) inhibited the EEG excitation effect caused by Na-PCN by increasing the power of δ1 and δ2 bands (P<0.01 vs model) and lowering the gravity frequency of PSD (P<0.01 vs model). JuA also remarkably reduced the Glu elevation induced by Na-PCN (P<0.05 vs model). Diazepam also depressed Glu concentration and lowered the gravity frequency, but it showed a different EEG pattern in increased β2-activity (P<0.01 vs model). EEG excitation caused by Na-PCN correlated with Glu elevation during the first hour. Neurophysiological inhibitory effects of JuA and diazepam were more persistent than their Glu inhibitoty effects.

Key words: Jujuboside A (JuA), Diazepam, Glutamate (Glu), Electroencephalogram (EEG), Hyperactivity, Penicillin sodium (Na-PCN), Power spectral density (PSD), Gravity frequency
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INTRODUCTION

Over excitation of nervous system can induce many kinds of diseases, like mania, hypochondriasis and epilepsy (Ribak, 1987). Although all anticonvulsants inhibit excessive neuronal activity, this acute effect appears to be produced by several mechanisms, which fall into three major categories: (1) blockade of voltage-gated sodium channels; (2) indirect or direct enhancement of inhibitory gamma-aminobutyric acid (GABA) neurotransmission; or (3) inhibition of excitatory glutamatergic neurotransmission (Soderpalm, 2002).

Excessive release of Glu is closely related to epilepsy (Bradford, 1995). Penicillin is a $GABA_A$ receptor blocker and is often used to induce epilepsy (Horn and Esseling, 1993; Fiacro *et al*., 1999; Uysal *et al*., 1996) and can also promote the release of Glu indirectly (Chen *et al*., 2001). Epileptiform activity and behavioural hyperactivity would develop immediately after Na-PCN was injected into the motor cortex or the olfactory cortex (El-Yamany and Horn, 2002; Horn *et al*., 1991).

In this work, a low dose of Na-PCN (1000 kIU/L, 3 μl) was introduced into the rat's left lateral ventricle (LV) to set up a hyperactivity model instead of an epilepsy model. Then cortical EEG and the Glu level in the ipsilateral hippocampus of rats were monitored in vivo to determine the excitatory degrees of rats. Influence of sedative drugs on this model was verified by co-administration of drug with Na-PCN in-

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tracerebroventricularly (icv). Diazepam, as a commonly used hypnotic, can remarkably inhibit the excitation effect induced by Na-PCN.

JuA is a main component of Jujubogenin extracted from the seed of *Ziziphus jujuba* Mill var *spinosa* (Bunge) Hu ex H F Chou (Rhamnaceae), which is widely used in Chinese traditional medicine for treating insomnia and anxiety (Shou *et al*., 2001). Our previous studies showed that JuA significantly decreased the slopes of excitatory postsynaptic potential (EPSP) and the amplitudes of the population spike (PS) in the first responses of granule cells and significantly decreased EPSP and PS in the responses of CA1 pyramidal cells (Shou *et al*., 2002). And JuA inhibited hippocampus Glu elevation in the first 20 min after its microinjection; and also inhibited the Glu-induced intracellular $[Ca^{2+}]_i$ increase, which may act through its anti-calmodulin action (Zhang *et al*., 2003). This study, by simultaneous EEG and Glu detection further examined the inhibitory effects of JuA on cortical EEG and hippocampal Glu of rats during the whole hour after icv drug administration, intended to explore the neurophysiological and neurochemical acting process of drugs simultaneously and the relation between EEG signals and Glu levels. The differences between the inhibitory effects of diazepam and JuA on EEG and Glu were also examined.

MATERIALS AND METHODS

Animals and chemicals

Experiments were performed on adult male Sprague-Dawley rats, obtained from Zhejiang Center of Laboratory Animals (Grade II, Certificate No. 2001001), weighing 240~300 g. JuA (purity 99%) was provided by the National Institute for the Control of Pharmaceutical and Biological Products; o-phthaldialdehyde and β-mercaptoethanol were obtained from Sigma; Urethane, penicillin sodium, diazepam injection solution and phenobarbital injection solution from China Medical Bioproducts Co.

Surgery

The rats were anaesthetized with urethane (1.25 g/kg, i.p.). Stainless steel electrodes were positioned epidurally on sensorimotor cortical areas for EEG recording. Intracerebral guide cannulas (BAS MD-2251, USA) were also implanted in advance to guide and secure probes. Bregma coordinates for EEG electrodes were *AP*=+1.2, *L*=±4.5, *H*=0; coordinates for hippocampus were *AP*=–5.8, *L*=+5.0, *H*=–3.0; coordinates for LV were *AP*=–0.8, *L*= +1.5, *H*=–3.5. Reference electrode was positioned epidurally on cerebellum (*AP*=–10, *L*=0, *H*=0). The guide canulla and electrodes were fixed to the skull with stainless steel screws and dental cement. The placement of the probes in the lateral ventricle and hippocampus is shown in Fig.1.

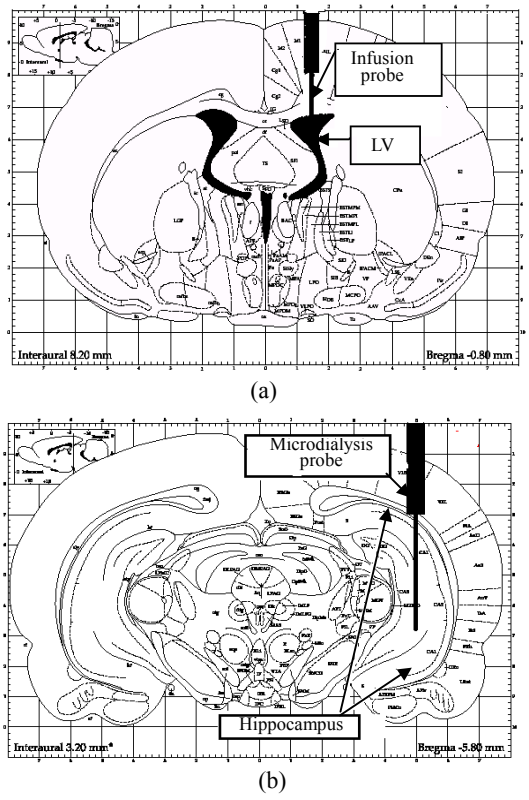

Fig.1 (a) Schematic coronal section of the rat brain (bregma=*P*–0.8) illustrating the placement of the infusion probe in the lateral ventricle; (b) Schematic coronal section of the rat brain (bregma=*P*–5.8) illustrating the placement of the microdialysis probe in hippocampus

EEG recording and signal processing

Five days was allowed for recovery. After settlement of the microdialysis probe and infusion probe (BAS MD-2252), a recording cable was connected to the EEG electrodes. EEG signals were amplified with a low-pass 100 Hz filter, digitized at 400 Hz by a PowerLab/4SP system (AD Instruments) with 12-bit

A/D, then were displayed and stored in a PC using the Chart program. These signals were re-filtered by an 8th order low-pass digital Butterworth filter with cut-off frequency of 40 Hz. Then the EEG data were analysed with a 4096-point, commonly used FFT algorithm, in 10 s epochs. This analysis was performed by a software designed by ourselves, which analysed 10 s epochs of data every 2 min (Su *et al*., 2003). Data were averaged during the total 20 min EEG simultaneously sampled with the collection of dialysate. Six frequency bands were used in analyses: $\delta 2$ (0.5–2 Hz), $\delta 1$ (2–4 Hz), θ (4–7.5 Hz), α (7.5–13.5 Hz), $\beta 1$ (13.5–20 Hz) and $\beta 2$ (20–30 Hz), absolute power spectrum density (PSD) in each of these frequency bands and gravity frequency of total bands were evaluated. Fig.2 is an example of the calculated results of a randomly chosen EEG signal segment.

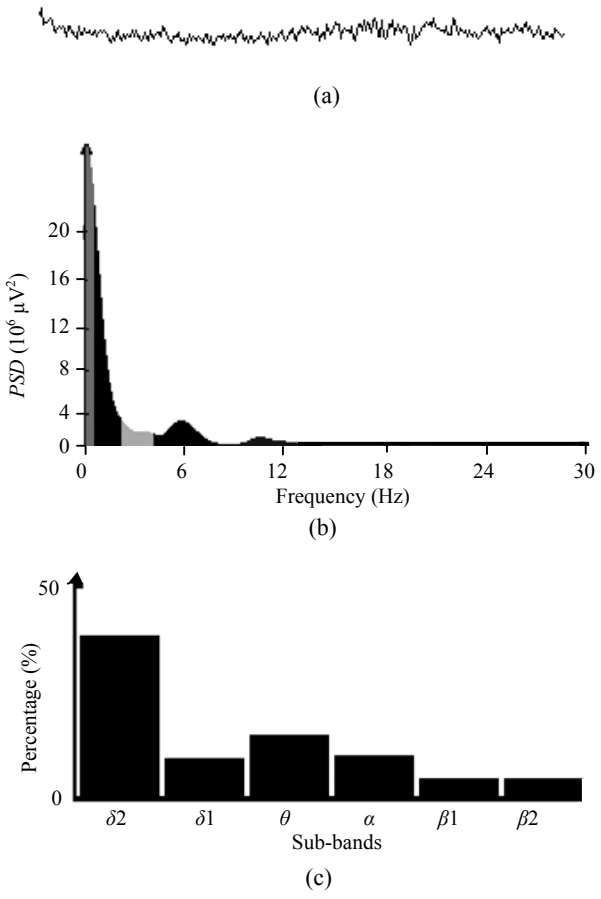

Fig.2 (a) A randomly chosen EEG signal segment; (b) PSD of the chosen EEG segment; (c) Histogram of PSD in six frequency bands

Microdialysis procedure

Microdialysis probe (BAS, MD-2204, 4 mm membrane) was inserted 120 min for equilibration before the experiment. Each experiment was processed by perfusing the probe with artificial cerebrospinal fluid (ACSF=126 mmol/L NaCl, 27.5 mmol/L $NaHCO_3$, 2.4 mmol/L KCl, 5 mmol/L KH_2PO_4, 5 mmol/L Na_2HPO_4, 0.5 mmol/L Na_2SO_4, 0.82 mmol/L $MgCl_2{\cdot}6H_2O$, 1.1 mmol/L $CaCl_2{\cdot}2H_2O$, 5 mmol/L glucose, and pH 7.4) at flow rate of 1 μl/min. Samples were manually collected (20 μl of dialysate per sample).

Estimation of Glu in the dialysates

The commonly used o-phthaldialdehyde (OPA)-β-mercaptoethanol precolumn derivatization, reversed-phase gradient elution and fluorescence detection (RF) method was modified and applied (Begley *et al*., 1994; Ye, 1988). The HPLC (Shimadzu-10AVP, Japan) employed two mobile phases: (1) buffer (0.1 mol/L KH_2PO_4, adjusted to pH 6.60): methanol=65:35, v/v; (2) buffer (ibid): methanol= 10:90, v/v. Separation was achieved on a C18 column (Hypersil, BDS, 5 μm, 4.0×200 mm). Each dialysate sample (20 μl) and 10 μl OPA derivating fluid were allowed to react for 1 min at room temperature before being injected onto the column through a 20 μl sample loop. The flow rate was 1 ml/min; EX: 357 nm; EM: 455 nm. The recovery of Glu was (105±9.8)%. In the range of 0.625~40 μmol/L, Glu concentration was linearly related with the peak area (r=0.9988). The detection limit for Glu in the dialysate was approximately 1 pmol/sample.

Hyperactivity model and its applications

The rats were randomly assigned to five groups: control group (solvent: ACSF), Na-PCN model group (1000 kIU/L Na-PCN), DZ group (0.1 g/L diazepam +1000 kIU/L Na-PCN), JuA1 group (0.05 g/L JuA+ 1000 kIU/L Na-PCN) and JuA2 group (0.1 g/L JuA+ 1000 kIU/L Na-PCN). In each group, six rats were studied.

Subsequently, samples were collected at 20 min intervals and the first sample collected after equilibration served as Glu baseline levels. At the beginning of the second stage, through the infusion probe placed in the LV, each rat was injected with 3 μl relevant drugs according to its group. Dialysates and

cortical EEG every 20 min in the first hour after microinjection of relevant drugs were analysed to evaluate the effects of the various drugs. The signs "-1", "-2" and "-3" represent the first, second and third 20 min after drug administration. For example, JuA1-1 means the first 20 min after icv drug injection in JuA1 group.

Histology and statistical analysis

Following completion of experiments, the rats were euthanized with urethane overdose and decapitated. The brains were cut into slices 50 μm wide by freezing-microtome (Microm, HM505E) and probe placements were verified for data presented in this study. Data presented as mean±*SD*. Statistical significance was evaluated with Student's *t*-test ($P<0.05$).

RESULTS

Effects of diazepam and JuA on EEG changes induced by Na-PCN

Every segment of 20 min cortical EEG signals of the corresponding dialysate was analyzed. The typical EEG signals in ACSF, Na-PCN, DZ, JuA1 and JuA2 groups are shown in Fig.3.

Analysis of the ipsilateral cortical EEG showed that when Na-PCN was applied, PSD in $\delta 2$ and $\delta 1$ bands decreased significantly; for example, the decreases in $\delta 2$ and $\delta 1$ bands were 0.17 dB and 0.14 dB in the first 20 min ($P<0.01$, Table 1). The gravity frequency of PSD increased from 6.50 Hz to 7.33 Hz ($P<0.01$) in the first 20 min after Na-PCN administration, then decreased to 6.97 Hz in the third 20 min (Table 1).

Analysis of the ipsilateral cortical EEG of diazepam group showed that PSD in $\delta 2$, $\delta 1$, θ, $\beta 1$ and $\beta 2$ bands all increased significantly compared with Na-PCN group; but the increases in low frequency bands $\delta 2$ (0.43 dB), $\delta 1$ (0.53 dB) and θ (0.33 dB) were greater than the increases in high frequency bands α (0.31 dB), $\beta 1$ (0.26 dB) and $\beta 2$ (0.09 dB) (DZ-1 for example, $P<0.01$, Table 1). At the same time, the gravity frequency of PSD decreased from 7.33 Hz to 6.18 Hz ($P<0.01$) in the first 20 min after diazepam administration; then it decreased to 5.95 Hz in the second 20 min and increased to 6.05 Hz in the last 20 min (Table 1).

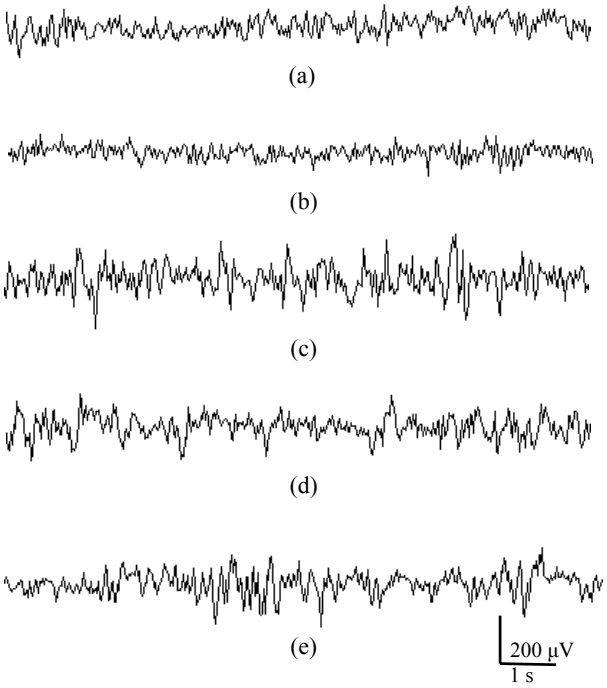

Fig.3 The rat cortical EEG signals of 10 s segments in the first 20 min after administration of different drugs
(a) ACSF; (b) Na-PCN; (c) DZ; (d) JuA1; (e) JuA2

Analysis of the ipsilateral cortical EEG of JuA group showed that PSD in low frequency bands $\delta 2$ and $\delta 1$ increased significantly compared with Na-PCN group; for example, $\delta 2$ increased 0.41 dB, $\delta 1$ increased 0.40 dB in JuA1-1; $\delta 2$ increased 0.38 dB, $\delta 1$ increased 0.39 dB in JuA2-1 ($P<0.01$, Table 2). Different from diazepam, JuA did not increase the PSD in high frequency bands $\beta 2$ of 20–30 Hz (Table 2) and the increases in $\beta 1$ and α were minor ($P<0.01$ vs DZ group). At the same time, the gravity frequency of PSD decreased significantly ($P<0.01$) during the whole hour after JuA1 and JuA2 administration ($P<0.01$, Table 2).

Effects of diazepam and JuA on Glu elevation induced by Na-PCN

The mean concentration of baseline Glu was 6.69±2.7 μmol/L. In model group, hippocampal Glu level was greatly elevated to 153% in the first 20 min compared with its baseline ($P<0.05$ vs control group), 196% in the second 20 min ($P<0.05$) and 120% in the third 20 min ($P>0.05$) (Fig.4). Accompanied by the increase of hippocampal Glu, rats were found in an over excitation state, beard erect and muscle tensed.

Table 1 The influence of diazepam (0.1 g/L) on Na-PCN (1000 kIU/L) induced cortical EEG power spectrum density (PSD) changes in six sub-bands and the gravity frequency of the total PSD (*n*=6)

Group	Baseline	ACSF	Na-PCN-1	Na-PCN-2	Na-PCN-3	DZ-1	DZ-2	DZ-3
Log(Power) (Log(μV^2), dB)								
$\delta 2$	6.65±0.30	6.60±0.23	6.48±0.20^c	6.43±0.29^c	6.55±0.29	6.91±0.30^f	6.91±0.33^f	6.96±0.31^f
$\delta 1$	6.56±0.29	6.52±0.25	6.42±0.22^b	6.42±0.28^c	6.52±0.31	6.95±0.31^f	6.88±0.38^f	6.99±0.31^f
θ	6.76±0.30	6.68±0.28	6.70±0.18	6.67±0.24	6.76±0.26	7.03±0.21^f	7.00±0.31^f	7.09±0.22^f
α	6.57±0.32	6.47±0.26	6.55±0.22	6.52±0.29	6.63±0.28^c	6.86±0.26^f	6.81±0.33^f	6.91±0.27^f
$\beta 1$	6.22±0.22	6.18±0.22	6.22±0.15	6.21±0.20	6.26±0.22^b	6.48±0.26^f	6.41±0.26^f	6.50±0.28^f
$\beta 2$	6.09±0.13	6.10±0.13	6.13±0.13	6.09±0.13	6.11±0.12	6.22±0.22^f	6.20±0.18^f	6.22±0.17^f
Gravity frequency (Hz)								
	6.36±1.16	6.50±0.77	7.33±0.78^c	7.32±0.84^c	6.97±0.74^c	6.18±0.88^f	5.95±0.81^f	6.05±0.69^f

$^b P<0.05$ vs ACSF; $^c P<0.01$ vs ACSF; $^f P<0.01$ vs Na-PCN Model; DZ-1 to Na-PCN-1, DZ-2 to Na-PCN-2, DZ-3 to Na-PCN-3. The signs "-1", "-2" and "-3" represent the first, second and third 20 min after drug administration

Table 2 The influence of JuA1 (0.05 g/L) and JuA2 (0.1 g/L) on Na-PCN (1000 kIU/L) induced cortical EEG power spectrum density (PSD) changes in six sub-bands and the gravity frequency of the total PSD (*n*=6)

Group	JuA1-1	JuA1-2	JuA1-3	JuA2-1	JuA2-2	JuA2-3
Log(Power) (Log(μV^2), dB)						
$\delta 2$	6.89±0.32^f	6.82±0.29^f	6.89±0.27^f	6.86±0.27^f	6.83±0.27^f	6.87±0.31^f
$\delta 1$	6.82±0.30^f	6.75±0.33^f	6.88±0.26^f	6.81±0.31^f	6.75±0.33^f	6.87±0.29^f
θ	6.90±0.23^f	6.90±0.22^f	6.97±0.26^f	6.94±0.28^b	6.87±0.23^f	6.95±0.21^f
α	6.74±0.24^f	6.68±0.34^f	6.80±0.22^f	6.72±0.34^f	6.65±0.36^b	6.76±0.22^f
$\beta 1$	6.33±0.20^f	6.30±0.17^f	6.39±0.22^f	6.34±0.19^f	6.27±0.19	6.35±0.15^b
$\beta 2$	6.13±0.13	6.12±0.13	6.15±0.17	6.17±0.16	6.11±0.14	6.15±0.11
Gravity frequency (Hz)						
	5.68±1.18^f	5.69±1.09^f	5.92±0.82^f	5.92±0.77^f	5.73±0.93^f	5.87±0.97^f

$^b P<0.05$ vs Na-PCN Model; $^f P<0.01$ vs Na-PCN Model; JuA1-1, JuA2-1 to Na-PCN-1, JuA1-2, JuA2-2 to Na-PCN-2, JuA1-3, JuA2-3 to Na-PCN-3; Data of ACSF and Na-PCN group are presented in Table 1. The signs "-1", "-2" and "-3" represent the first, second and third 20 min after drug administration

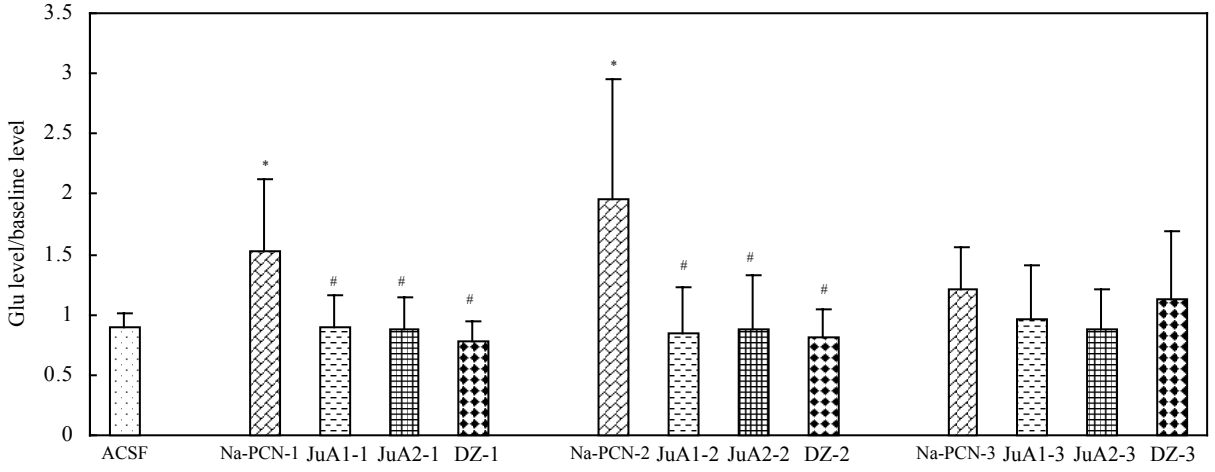

Fig.4 Influence of JuA1 (0.05 g/L), JuA2 (0.1 g/L) and DZ (0.1 g/L) on the increase in extracellular level of Glu induced by 1000 kIU/L Na-PCN in rat hippocampus in vivo. JuA were co-injected with Na-PCN

Values are means±*SD*, *n*=6; $^* P<0.05$ vs ACSF; $^\# P<0.05$ vs Na-PCN Model. JuA1-1, JuA2-1 and DZ-1 to Na-PCN-1, JuA1-2, JuA2-2 and DZ-2 to Na-PCN-2, JuA1-3, JuA2-3 and DZ-3 to Na-PCN-3. The signs "-1", "-2" and "-3" represent the first, second and third 20 min after drug administration

When 0.1 g/L diazepam was co-injected into the LV with Na-PCN, the increase of hippocampal Glu was inhibited. The Glu concentrations were 0.78±0.16 and 0.82±0.23 times baseline in the first two 20 min (P<0.05 vs Na-PCN-1 and Na-PCN-2 respectively, Fig.4). Diazepam inhibited the excitatory effect of Na-PCN completely by reducing Glu level below its baseline at this stage. When 0.05 g/L or 0.1 g/L JuA was co-injected into the LV with Na-PCN, the increase of hippocampal Glu was inhibited. In 0.05 g/L JuA (JuA1) group, the Glu concentrations decreased 41.8% (P<0.05), 57.0% (P<0.05) and 20.5% (P>0.05) compared with Na-PCN group respectively in three time courses (Fig.4). In 0.1 g/L JuA (JuA2) group, the Glu concentrations decreased 43.1% (P<0.05), 54.9% (P<0.05) and 27.2% (P>0.05) compared with Na-PCN group respectively in three time courses (Fig.4). In diazepam and JuA group, when drug was injected, rats got very excited for the first several minutes, indicating an over excitation state; right after that, inhibition appeared and the rats became drowsy.

DISCUSSION

We studied the effects of Na-PCN on cortical EEG and hippocampal Glu, and examined the effects of diazepam and JuA on Na-PCN induced over excitation by hippocampal Glu and cortical EEG simultaneous detection and PSD analysis.

Our results showed that 1000 kIU/L Na-PCN induced hyperactivity in rats by greatly increasing Glu level in hippocampus and enhancing the gravity frequency of power spectra density. This change of gravity frequency was mainly due to the power decrease in low frequency bands δ2 and δ1. Glu remained at a high level in the first two 20 min after Na-PCN administration, then declined in the third 20 min. This tendency was well reflected in the EEG power spectra density. The PSD of low frequency bands δ2 and δ1 deceased remarkably in the first two 20 min, then rose to their control level in the third 20 min. The corelation between cortical EEG and hippocampal Glu can help us to understand more deeply the mechanism of excitatory effects of Na-PCN on central nervous system.

Furthermore, we observed that diazepam (0.1 g/L) could inhibit the hyperactivity induced by Na-PCN and effectively reduced the induced elevation of hippocampal Glu. It is known that benzodiazepine receptor and $GABA_A$ receptor compose a benzodiazepine-GABA receptor-ionophore complex. A variety of centrally acting anxiolytic, depressant and anticonvulsant drugs bind to one of the sites in the complex and modulate the binding of ligands at the other sites (Ticku, 1983). As a benzodiazepine, diazepam may reduce Glu indirectly by potentiating GABA ergic neurotransmission at $GABA_A$ receptors via a modulatory binding site. When two different concentrations of JuA were respectively co-injected with Na-PCN, the increase in extracellular levels of Glu was also inhibited. Diazepam (0.05 g/L JuA and 0.1 g/L JuA) inhibited Glu significantly (P<0.05) in the first two 20 min after Na-PCN administration, then their Glu-inhibition effect was depressed in the next 20 min. However, in EEG analysis, the inhibition effects, including δ2, δ1, θ enhancement and gravity frequency decline, continued in the next 20 min. This phenomenon suggested that the neural electrophysiology inhibitory effect of these two sedative drugs was more persistent than their Glu inhibitoty effect. Investigation on the corelation between EEG signals and other neurotransmitters, like NE, DA and 5-HT, will help to shed more light on the action mechanism of diazepam and JuA.

Although both diazepam and JuA greatly reduced the gravity frequency of PSD, their influence on specific frequency bands was quite different. Diazepam greatly increased power spectra density in all δ2, δ1, θ, β1 and β2 bands, but JuA (0.05 g/L and 0.1 g/L) did not increase power spectra density in β2 band, and the increase in β1 was minor. It was reported that though being a sedative, diazepam increases high frequency β acticity in EEG, as confirmed in this experiment (Marijtje *et al*., 2000). This increase of β activity implies that some special neurons of rat cortex were excited though generally the brain was in an inhibitory condition. The possibility of EEG β activity enhancement related to the side effect of diazepam had been discussed (Kozhechkin, 2003). To verify it, further study is needed to be done, which may help to reveal more implicit advantages of the Chinese medicine JuA over diazepam.

Na-PCN is an agitator always used to increase the combination of transmitter-receptor of Glu on postsynaptic membrane and result in the calcium ion

influx that can trigger the burst firing (Futamachi and Prince, 1975). Our previous studies showed the converse effect of JuA to Na-PCN may be due to its effect of suppressing Glu release from presynaptic terminals through inhibiting the activity of CaM, which will also interrupt the signal processing triggered by the NMDA mediated calcium influx in the paired-pulse facilitation (Shou *et al*., 2002; Zhang *et al*., 2003). In the present study, we further proved that JuA has inhibitory effects on cortical EEG by increasing the power of δ1 and δ2 bands and lowering the gravity frequency of PSD, but its action mechnism is different from that of diazepam as shown in the EEG β2 acticity. And the neurophysiological inhibitory effect of JuA was more persistent than its Glu inhibitoty effect. To reveal its mechanism, further investigation on the correlation between EEG signals and other neurotransmitters shall be done.

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