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Inhibition of Evoked Glutamate Release by Neurosteroid Allopregnanolone Via Inhibition of L-Type Calcium Channels in Rat Medial Prefrontal Cortex

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Allopregnanolone is one of the most important neurosteroids in the brain. We studied the effect and mechanism of allopregnanolone on spontaneous and evoked glutamate release in the medial prefrontal cortex using electrophysiological and biochemical methods combined with pharmacological approaches. The results showed that allopregnanolone had no effects on the frequency of miniature excitatory postsynaptic current (mEPSCs), but inhibited the depolarizing agent veratridine-evoked increase in the frequency of spontaneous excitatory postsynaptic currents (sEPSCs) and inhibited the first of the two responses evoked by a pair of electrical pulses more effectively than the second, resulting in increased paired-pulse facilitation (PPF) and thus suggesting a presynaptic inhibitory effect on electrical pulse-evoked glutamate release. A similar effect was also obtained for the effect of allopregnanolone on protein kinase A (PKA) activation, an upstream event of presynaptic glutamate release. Interestingly, allopregnanolone had none of these effects in the striatum. In the study of the upstream mechanism of the PKA inhibition by allopregnanolone, we found that allopregnanolone inhibited extracellular calcium influx-evoked PKA activation, but had no effects on intracellular calcium store release-evoked PKA activation; L-type calcium channel antagonists, but not N- and P/Q-type calcium channel antagonist, blocked the effect of allopregnanolone; allopregnanolone inhibited L-type calcium channel agonist-evoked increase in the PKA activity, intrasynaptosomal calcium concentration and frequency of sEPSCs. These results suggest that allopregnanolone inhibits evoked glutamate release via the inhibition of L-type calcium channels in the medial prefrontal cortex, but does not in the striatum.
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INTRODUCTION

Recent evidence suggests that neurosteroid allopregnanolone may have profound psychotropic effects. Intraventricular application of allopregnanolone induced catalepsy in mice (Khisti *et al*, 1998) and reduced conditioned avoidance, apomorphine-induced cage climbing and amphetamine-induced motor hyperactivity in rodents (Khisti *et al*, 2002), which all were important indexes to predict an agent having neuroleptic property (Khisti *et al*, 1998, 2002). Treatment with atypical neuroleptic drugs such as clozapine or olanzapine, but not haloperidol, has been shown to induce a rapid and marked increase in the concentration of allopregnanolone in the brain (Marx *et al*, 2003, 2000; Barbaccia *et al*, 2001). However, the mechanism underlying the psychotropic effect of allopregnanolone still requires to be studied.

One mechanism for the psychotropic effect of allopregnanolone has been proposed to be mediated by its potentiating effect on GABAergic neurotransmission because it has been shown that allopregnanolone is a potent positive endogenous allosteric modulator of the GABA receptor (Majewska *et al*, 1986) and clozapine and olanzapine increase cerebral cortical allopregnanolone concentration in rats to levels known to affect GABAergic neurotransmission (Marx *et al*, 2000, 2003). However, maybe other mechanisms, in addition to the potentiation of GABAergic neurotransmission, are also involved in the psychotropic effect of allopregnanolone. Here, we propose that the actions of allopregnanolone on glutamatergic neurotransmission, especially on presynaptic glutamate release in the medial prefrontal cortex, may constitute another mechanism for the psychotropic effect of allopregnanolone because the medial prefrontal cortex has been well known to play important roles in cognition and neuropsychiatric processes (Castner *et al*, 2004; Seamans *et al*, 1995)

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and the glutamate-mediated synaptic inputs to the pyramidal cells of the layers V–VI of the medial prefrontal cortex are very important for the function of the medial prefrontal cortex (Ishikawa and Nakamura, 2003; Nelson *et al*, 2002; Hempel *et al*, 2000). Moreover, abnormally enhanced presynaptic glutamate release in the medial prefrontal cortex has been reported to play an important role in the pathophysiology of neuropsychiatric diseases (Adams and Moghaddam, 1998; Moghaddam *et al*, 1997; Moghaddam and Adams, 1998). However, it is still not clear whether allopregnanolone has actions on the presynaptic glutamate release in the medial prefrontal cortex. Therefore, in the present paper, we studied the effect of allopregnanolone on the basal and evoked presynaptic glutamate release in the medial prefrontal cortex by examining the effect of allopregnanolone on the frequency of miniature excitatory postsynaptic currents (mEPSCs), on the depolarizing agent-evoked increase in the frequency of spontaneous excitatory postsynaptic currents (sEPSCs) and on the paired-pulse facilitation (PPF) evoked by a pair of electrical pulses with whole-cell patch clamp recording method in rat slices in the presence of the $GABA_A$ receptor antagonist picrotoxin. We also studied the mechanism of the effect of allopregnanolone using electrophysiological and biochemical methods combined with pharmacological approaches and made a comparison for the effect of allopregnanolone in the cognition-related brain region—the medial prefrontal cortex and the movement-related brain region—the striatum.

MATERIALS AND METHODS

Preparation of Slices

Sprague–Dawley rats (20- to 30-day-old) were anesthetized with chloral hydrate (400 mg/kg, i.p.). All experimental procedures conformed to Fudan University as well as international guidelines on the ethical use of animals and all efforts were made to minimize the number of animals used and their suffering. Brain slices were prepared according to procedures described previously (Wang and Zheng, 2001). Serial coronal slices (300 μm) were cut and transferred to an incubating chamber (30–32°C) where they stayed for at least 1 h before recordings began.

Whole Cell Recording

The slice was continuously perfused with artificial cerebrospinal fluid (ACSF), containing 130 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 2 mM $MgSO_4$, 1.25 mM NaH_2PO_4, 26 mM $NaHCO_3$, 10 mM glucose, 10 mM sucrose, and saturated with 95%O_2/5%CO_2. Cells were visualized with an infrared-DIC microscope (Olympus BX50WI) and a CCD camera. Pyramidal cells were identified by their pyramidal shape, large soma, and presence of apical dendrites. Electrodes were pulled from glass capillaries using a Narishige micropipetter puller (model PB-7; Narishige, Japan). They were filled with a solution containing 140 mM KGluconate, 0.1 mM $CaCl_2$, 2 mM $MgCl_2$, 1 mM EGTA, 2 mM ATP.K_2, 0.1 mM GTP.Na_3, and 10 mM HEPES (pH 7.25) and had a resistance of 4–6 MΩ. Voltage and current signals were recorded with Axopatch 200B amplifier (Axon, Union City, USA) connected to a Digidata1200 interface (Axon, Union City, USA). The Data were digitized and stored on disks using pClamp (version 6; Axon, Union City, USA). Resting membrane potential and action potentials were recorded under the current clamp mode. mEPSCs and sEPSCs were recorded in sweeps of 2 s at a holding potential of −70 mV under the voltage clamp mode in the presence of picrotoxin (50 μM) and TTX (1 μM, except for sEPSCs). To evoke EPSCs, a bipolar stimulating electrode was placed in layer V, 10–50 μm laterally to the apical dendrite of the recorded cell. The EPSCs were elicited by stimulating pulses with the stimulation intensity adjusted to evoke an EPSC that was ∼30% of the maximum amplitude at a holding potential of −70 mV and in the presence of picrotoxin (50 μM) to block $GABA_A$ receptors. Paired pulse facilitation (PPF) was induced by a pair of stimuli given at short intervals (50 ms) at 0.05 Hz. To induce NMDA or AMPA currents, NMDA (50 μM) or AMPA (20 μM) was pressure ejected from a pipette positioned near the soma of recorded cells. NMDA currents were recorded under the voltage clamp mode at a holding potential of −60 mV and in the presence of TTX (1 μM) to block Na^+ currents, picrotoxin (50 μM) to block $GABA_A$ receptors and zero Mg^{2+} to reveal NMDA responses. AMPA currents were recorded in a way similar to that for NMDA except for 2 mM Mg^{2+} in ACSF. The series resistance (Rs) was monitored by measuring the instantaneous current in response to a 5 mV voltage step command. Series resistance compensation was not used, but cells where Rs changed by >15% were discarded.

Synaptosome Preparation and PKA Activity Assay

Synaptosomes were prepared from the medial prefrontal cortex and the striatum of Sprague–Dawley rats as described previously (Harrison *et al*, 1988). PKA activity was assessed with PepTag Non-Radioactive cAMP-Dependent PKA Assay Kit from Promega according to the manufacturer's instruction. The assay was based on the change in the net charge of the fluorescent PKA substrate before and after phosphorylation. The change in the net charge of the substrate allowed the phosphorylated and nonphosphorylated version of the substrate to be rapidly separated on an agarose gel at neutral pH. The intensity of the fluorescence of phosphorylated peptides reflected the activity of PKA (Lou and Pei, 1997).

Intrasynaptosomal [Ca^{2+}] Measurement

Intrasynaptosomal Ca^{2+} levels were measured using the fluorescent indicator Fluo-3/acetoxymethyl ester (Fluo-3AM) as described previously for synaptosomes (Minta *et al*, 1989). Fluorescence changes were obtained at 527 nm in response to 485 nm excitation. Maximum and minimum ratios were determined using 1 mM Ca^{2+} and 5 mM EGTA plus 40 mM Tris base, respectively, in samples lysed at the end of each experiment with 0.3% Triton X-100 (final). Free intrasynaptosomal [Ca^{2+}] was calculated from the ratio data according to the equations described by Grynkiewicz *et al* (1985), using a K_d value of 400 nM for binding of Ca^{2+} to Fluo-3AM (Nichols and Mollard, 1996; Nichols *et al*, 1994).

Off-Line Data Analysis

Off-line data analysis was performed using Mini Analysis Program (Synaptosoft), SigmaPlot (Jandel Scientific) and Origin (Microcal Software Inc.). The record of mEPSCs and sEPSCs was shown with high time resolution and the events that did not show a typical EPSC waveform were rejected manually. The frequency, inter-event intervals and amplitude of mEPSCs and sEPSCs were measured. In most cases, >100 mEPSCs and sEPSCs were collected under control conditions as well as for each pharmacological condition. In time course experiments of mEPSCs and sEPSCs, the frequency mEPSCs and sEPSCs was measured over a 30 s interval and it was defined as mini frequency. Numerical data were expressed as mean $\pm$ SEM (standard errors of means). Statistical significance was determined using either Kolmogorov–Smirnov test (K–S test) for comparison of mEPSC distributions, or Student's paired *t*-test (unless otherwise stated) for comparison between two groups or ANOVA followed by Student–Newman–Keuls test for comparisons between three or more groups (unless otherwise stated). In all cases *n* refers to the number of cells studied.

Drugs

Picrotoxin, ryanodine, veratridine, allopregnanolone (5α-pregnan-3α-ol-20-one), KGluconate, ATP.K_2, GTP.Na_3, Bay k8644 (S-(−)-1,4-dihydro-2,6-dimethyl-5-nitro-4-(2-[trifluoromethyl]phenyl)-3-pyridine carboxylic acid methyl ester), forskolin, H89 (*N*-[2-(p-bromocinnamylamino)-ethyl]-5-isoquinoline-sulfonamide 2HCl), nimodipine, pluronic F-127, Fluo-3AM, verapamil, NMDA (*N*-methyl-D-aspartate), and AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) were purchased from Sigma. PepTag assay kit for non-radioactive detection of PKA was from Promega Corporation. TTX (tetrodotoxin) was made in the Research Institute of Aquatic Products of Hebei, PR China. Percoll was purchased from Amersham Biosciences Corporation. ω-Agatoxin TK and ω-conotoxin MVIIA were purchased from Alomone Labs Ltd. Other reagents in AR grades were products of Shanghai Chemical Plant. All drugs were dissolved in dH_2O, except for picrotoxin, allopregnanolone, Bay k8644 ryanodine, nimodipine and forskolin, which were dissolved in DMSO. When DMSO was used as vehicle, drugs were initially dissolved in 100% DMSO and then diluted into ACSF at a final DMSO concentration lower than 0.1%. In vehicle control experiments, we confirmed that the final concentration of DMSO in ACSF had no detectable effects on the parameters we observed. Allopregnanolone, ryanodine, TTX, picrotoxin, forskolin, H89, Bay k8644, verapamil, and veratridine were applied by bath perfusion. NMDA and AMPA were pressure ejected from a local pipette.

RESULTS

Allopregnanolone has no Effects on the Frequency of mEPSCs and sEPSCs, but Inhibits Veratridine-Evoked Increase in the Frequency of sEPSCs and Increases PPF

To investigate if allopregnanolone has a modulatory effect on action potential-independent glutamate release in the pyramidal cells of the layers V–VI of the medial prefrontal cortex, we have observed the effect of allopregnanolone on the frequency of mEPSCs, which is thought to result from the release of glutamate in an action potential-independent manner and has been used as a measure of modulation of this kind of release (Bouron and Reuter, 1999; Evans *et al*, 2000; Iyadomi *et al*, 2000; Schoppa and Westbrook, 1997). Typical records illustrating the effect of allopregnanolone (20 μM) on the frequency of mEPSCs were shown in Figure 1a. From these raw data, it seemed that allopregnanolone had no apparent effects on the frequency of mEPSCs. To demonstrate it in a more accurate manner, we constructed cumulative probability plots with the use of the inter-event interval distribution of mEPSCs recorded before and after allopregnanolone and made a comparison between the distribution in control and allopregnanolone with K–S test. The result showed that allopregnanolone (20 μM) had no effects on the cumulative distribution of the inter-event intervals of mEPSCs ($P>0.05$, K–S test, Figure 1b). We repeated the experiment in six cells and obtained a similar result. The averaged time course curve also showed that the change after allopregnanolone was not apparent (Figure 1c). Figure 1d was the averaged frequency before and during 5–10 min after allopregnanolone (5.9 ± 1.6 Hz before and 5.8 ± 0.7 Hz during 5–10 min after allopregnanolone, $n=6$), showing no statistically significant changes after allopregnanolone ($P>0.05$).

To study the effect of allopregnanolone on depolarization-evoked presynaptic glutamate release, we used veratridine, a chemical depolarizing agent (Ulbricht, 1969) and a commonly used strategy to depolarize neurons (Lopez *et al*, 2001; Garcia-Sanz *et al*, 2001), to induce an increase in the frequency of sEPSCs and then observed the effect of allopregnanolone on this increase. As shown in Figure 2a, veratridine (4 μM) alone evoked a significant increase in the frequency of sEPSCs ($n=6$), but in the presence of allopregnanolone (20 μM), the veratridine-evoked increase was inhibited (Figure 2c, $n=6$). The averaged frequency before and during 5–10 min after veratridine in the absence and presence of allopregnanolone also showed that the veratridine effect (Figure 2b, $n=6$, $P<0.05$) was inhibited by allopregnanolone (Figure 2d, $n=6$, $P>0.05$), while allopregnanolone had no effects on the basal frequency of sEPSCs ($n=6$, data not shown). We also checked the concentration-dependence of the inhibitory effect of allopregnanolone on the veratridine-evoked increase in the frequency of sEPSCs. The result showed that the effect of allopregnanolone increased with an increase in concentration and appeared to reach a plateau after 0.5 μM (Figure 3, $n=4$–7). Interestingly, allopregnanolone (20 μM) had no influences on the veratridine-evoked effect in the spiny neurons of the striatum (Figure 2e–h, $n=6$).

To test the effect of allopregnanolone on electrical stimulus-evoked presynaptic glutamate release, we have observed the effect of allopregnanolone on PPF, which is measured as the ratio of EPSC amplitude in response to two successive stimulation pulses and is a frequently used parameter to monitor electrical stimulus-evoked presynaptic glutamate release (Martin and Buno, 2003; Clark *et al*, 1994; Creager *et al*, 1980; Zucker, 1973). The results showed that under control conditions the amplitude of the second EPSC was larger than that of the first EPSC, indicating that

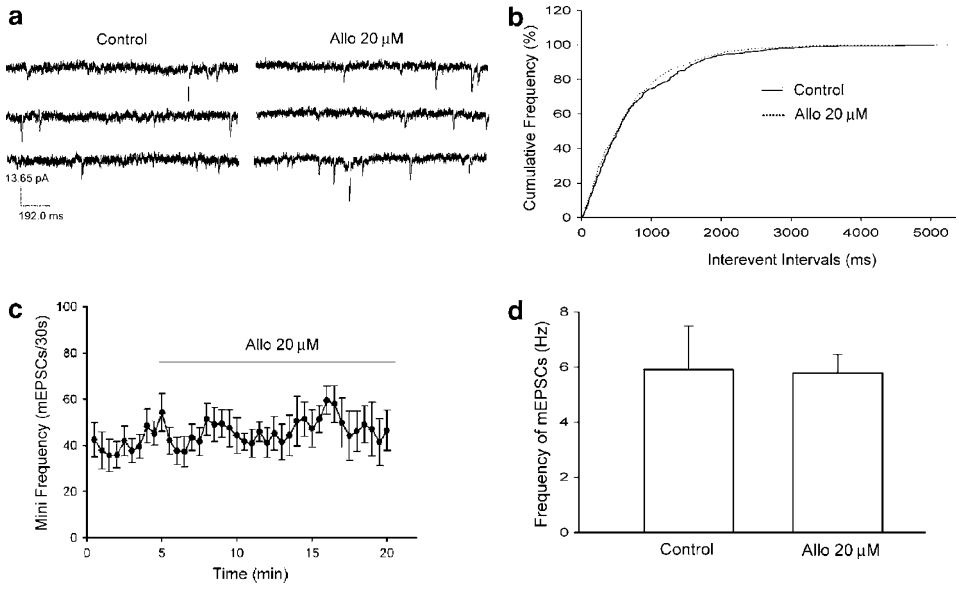

Figure 1 Effect of allopregnanolone (Allo) on the frequency of mEPSCs in the pyramidal cells of the layers V–VI of the medial prefrontal cortex. (a) Typical records in control and during application of Allo 20 μM. Holding potential: −70 mV. (b) The cumulative probability plots with the use of the inter-event interval distribution of mEPSCs before and after Allo 20 μM. ($P>0.05$, K–S test). (c) The time course of the change after Allo 20 μM. (d) The averaged result before and after Allo 20 μM ($P>0.05$, $n=6$).

the second synaptic response was facilitated by the first EPSC (Figure 4a). After the addition of allopregnanolone (20 μM), the first EPSC was inhibited by 64.1 ± 16.5% ($n=5$), but the second synaptic response only by 32.9 ± 9.1% (Figure 4b, $n=5$). Therefore, PPF was increased after allopregnanolone. The averaged PPF was increased from 42.2 ± 9.7% before to 131.5 ± 40.7% at 10 min after allopregnanolone (Figure 4c, $n=5$, $P<0.05$). This result suggests that allopregnanolone also inhibits electrical stimulus-evoked presynaptic glutamate release.

We also evaluated the effect of allopregnanolone on postsynaptic NMDA and AMPA receptors. To induce NMDA or AMPA currents, NMDA (50 μM) or AMPA (20 μM) was pressure ejected from a pipette positioned near the soma of recorded cells. NMDA currents were recorded under the voltage clamp mode at a holding potential of −60 mV in the presence of TTX (1 μM), picrotoxin (50 μM) and zero Mg^{2+}. AMPA currents were recorded in a way similar to that for NMDA except for 2 mM Mg^{2+} in ACSF. The results showed that allopregnanolone (20 μM) had no significant effects on NMDA and AMPA currents. The averaged NMDA currents before and after allopregnanolone was 51.0 ± 4.0 and 52.6 ± 3.9 pA ($n=6$, $P>0.05$) and the averaged AMPA currents before and after allopregnanolone was 35.2 ± 1.2 and 40.5 ± 10.2 pA ($n=6$, $P>0.05$). These results were consistent with those reported by Leskiewicz *et al* (1998) who showed that allopregnanolone at concentrations of 0.001–100 μM did not affect the binding of [^{3}H]-AMPA and [^{3}H]-MK-801 to AMPA and NMDA receptors.

Allopregnanolone Inhibits Veratridine-Evoked PKA Activation

It has been known that PKA activation is an important upstream event of presynaptic glutamate release (Leenders and Sheng, 2005; Millan *et al*, 2003; Grilli *et al*, 2004). This statement was consistent with our results that veratridine (4 μM) could evoke presynaptic PKA activation (data shown later) and the PKA inhibitor H89 (1 μM) could block the veratridine-induced increase in the frequency of sEPSCs ($n=6$, data not shown). Therefore, we hypothesize that allopregnanolone may have an inhibitory effect on the veratridine-evoked PKA activation and this inhibition may be a major mechanism for the allopregnanolone-mediated inhibition of evoked glutamate release. To test this hypothesis, we observed the effect of allopregnanolone on the veratridine-evoked PKA activation in the presence of the $GABA_A$ receptor antagonist picrotoxin (200 μM) in synaptosomes of the medial prefrontal cortex. As shown in Figure 5a, an addition of allopregnanolone (20 μM) in the milieu had no effects on basal PKA activity, but it could significantly inhibit the veratridine-evoked PKA activation. PKA activity was enhanced to 155.06 ± 10.6% of control level by veratridine (4 μM) without allopregnanolone, but in the presence of allopregnanolone the effect of veratridine was only 111.5 ± 13.9% of control level, showing that the effect of veratridine was significantly inhibited by allopregnanolone ($n=4$, $P<0.05$, compared to veratridine alone group). Interestingly, here we also checked the effect of allopregnanolone on the veratridine-evoked PKA activation in synaptosomes of the striatum and obtained a similar result to that of the above-mentioned electrophysiological experiment, that is, allopregnanolone had no effects on the veratridine-evoked PKA activation in the striatum (Figure 5b, $n=5$).

To check if allopregnanolone had a direct effect on the activated PKA in the medial prefrontal cortex, we observed the influence of allopregnanolone on the PKA agonist forskolin-evoked increase in the PKA activity. The result showed that allopregnanolone had no influence on the forskolin-evoked increase in the PKA activity. The averaged

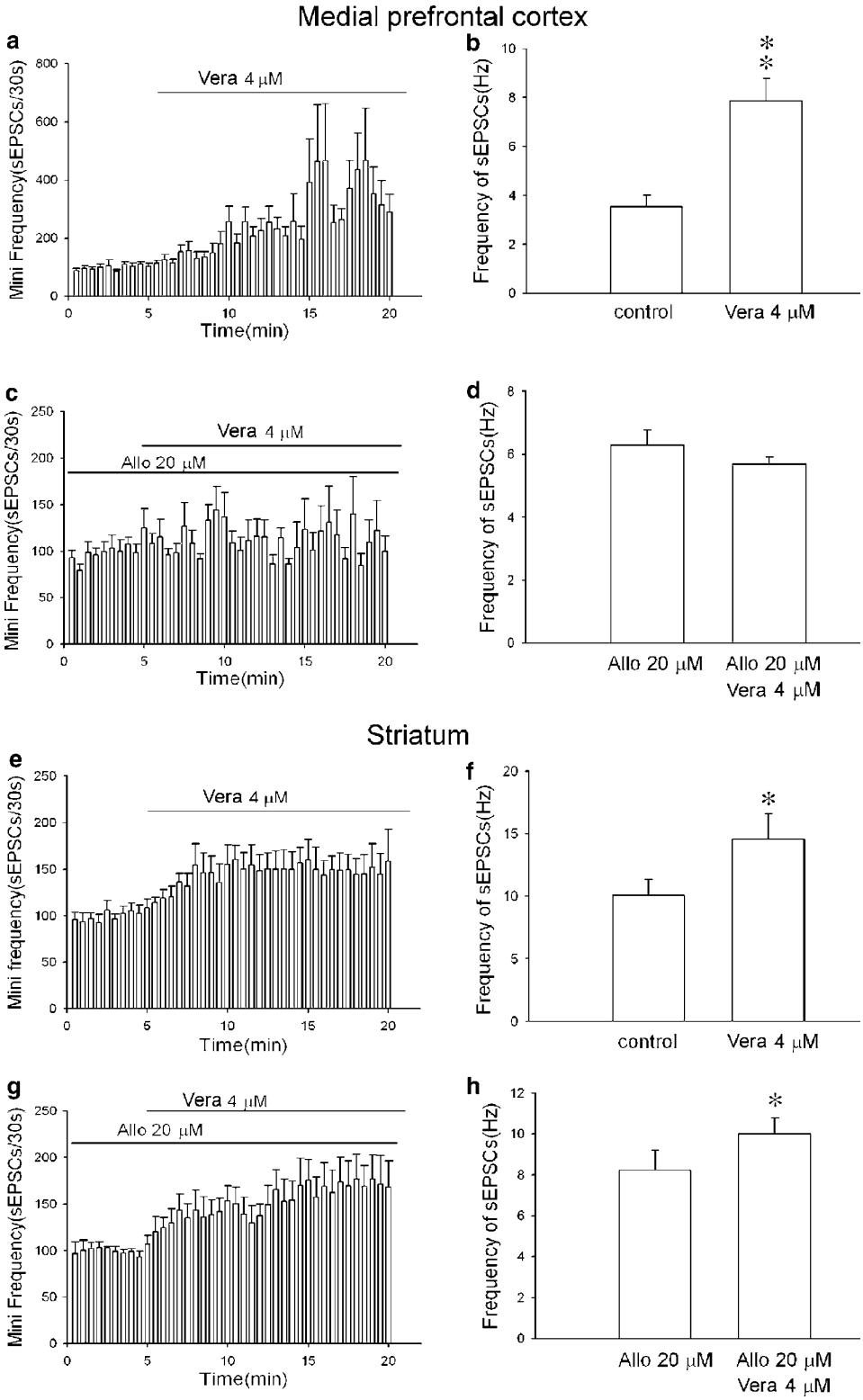

Figure 2 Effect of allopregnanolone (Allo) on the depolarizing agent veratridine (Vera)-evoked increase in the frequency of sEPSCs. Left panels: time course plot in a group of cells. Right panels: the averaged result in a group of cells. (a, b) Effect of Vera (4 μM) on the frequency of sEPSCs in the pyramidal cells of the layers V–VI of the medial prefrontal cortex in the absence of Allo. $n = 6$, $^{**}P < 0.01$. (c, d) Effect of Vera (4 μM) on the frequency of sEPSCs in the pyramidal cells of the layers V–VI of the medial prefrontal cortex in the presence of Allo (20 μM). $n = 6$. (e, f) Effect of Vera (4 μM) on the frequency of sEPSCs in the spiny neurons of the striatum in the absence of Allo. $n = 6$, $^{*}P < 0.05$. (g, h) Effect of Vera (4 μM) on the frequency of sEPSCs in the spiny neurons of the striatum in the presence of Allo. $n = 6$, $^{*}P < 0.05$.

increase percentage of PKA activity evoked by forskolin (20 μM) without and with allopregnanolone (20 μM) was 208.6 ± 26.5 and 204.4 ± 13.2% of control level, respectively, showing that the effect of forskolin was not influenced by allopregnanolone ($n = 4$, $P > 0.05$). We also tested the influence of allopregnanolone on the PKA agonist forskolin-evoked increase in the frequency of sEPSCs and obtained a similar result to the PKA experiment ($n = 6$, data not shown).

Allopregnanolone Inhibits Extracellular Calcium Influx-Evoked PKA Activation, but has no Effects on Intracellular Calcium Store Release-Evoked PKA Activation

To test if allopregnanolone had an inhibitory effect on extracellular calcium influx-evoked PKA activation through calcium channels, here we used another depolarizing agent-high K^+ that was only calcium channel-dependent (Sitges and Galindo, 2005; Lingamaneni and Hemmings, 1999), rather than veratridine that was both sodium channel- and calcium channel-dependent (Sitges and Galindo, 2005; Lingamaneni and Hemmings, 1999), to induce the extracellular calcium influx-evoked PKA activation. In the meantime, we used high concentration of ryanodine (10 μM) to block intracellular calcium release from the endoplasmic reticulum (Meissner, 1986). Under these conditions, we observed the effect of allopregnanolone on the PKA activation. The result showed that high K^+ (15 mM) could increased the PKA activity and allopregnanolone (20 μM) significantly inhibited the high K^+-evoked PKA activation (left panel of Figure 6a). PKA activity was enhanced to 182.8 ± 15.1% of control level by high K^+ without allopregnanolone, but in the presence of allopregnanolone the effect of high K^+ was only 125.0 ± 6.4% of control level, showing that the effect of high K^+ was significantly inhibited ($n = 4$, $P < 0.05$, compared to high K^+ alone group). We also observed the effect of allopregnanolone on the intracellular calcium store release-evoked PKA activation, but did not find significant effect. As shown in the right panel of Figure 6a, low concentration of ryanodine (1 μM), which had been reported to be able to promote intracellular calcium release from the endoplasmic reticulum (Meissner, 1986), significantly increased the PKA activity ($n = 4$, $P < 0.05$) and allopregnanolone (20 μM) had no significant influence on the ryanodine-evoked PKA activation. PKA activity was enhanced to 135.5 ± 7.8% of control level by ryanodine alone and in the presence of allopregnanolone the PKA activity was still enhanced to 136.4 ± 8.8% of control level by ryanodine, showing that the effect of ryanodine in the presence of allopregnanolone did not change significantly. We also tested the influence of allopregnanolone on the high K^+- and ryanodine-evoked increase in the frequency of sEPSCs and obtained a similar result to the PKA experiments (Figure 6b, $n = 5$–6).

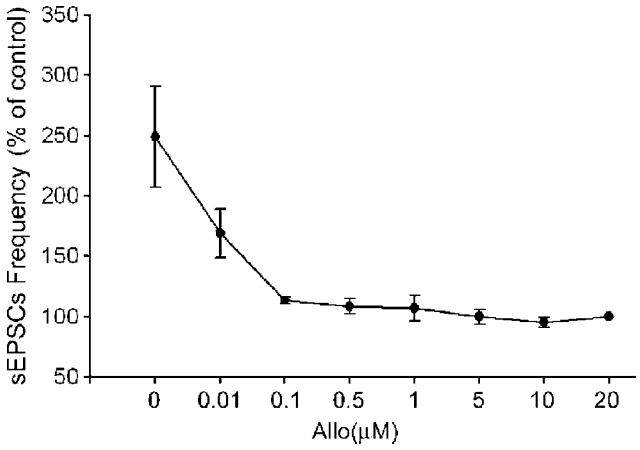

Figure 3 The concentration-dependence of the inhibitory effect of allopregnanolone (Allo) on the veratridine-evoked increase in the frequency of sEPSCs in the pyramidal cells of the layers V–VI of the medial prefrontal cortex. $n = 4$–7.

L-Type Calcium Channel Antagonists, but not N- and P/Q-Type Calcium Channel Antagonist, Block the Effect of Allopregnanolone

To check the possible contribution of L-type calcium channel inhibition to allopregnanolone-mediated inhibition of the high K^+-evoked PKA activation, we observed the influence of L-type calcium channel antagonist verapamil on the effect of allopregnanolone. Application of verapamil (50 μM) caused a suppression of the high K^+-evoked increase in PKA activity by 18.1 ± 4.6% (from 157.2 ± 11.1 to 128.2 ± 9.8% of control, $n = 4$) and completely blocked the effect of allopregnanolone (Figure 7a, $n = 4$). The averaged allopregnanolone (20 μM)-induced decrease in

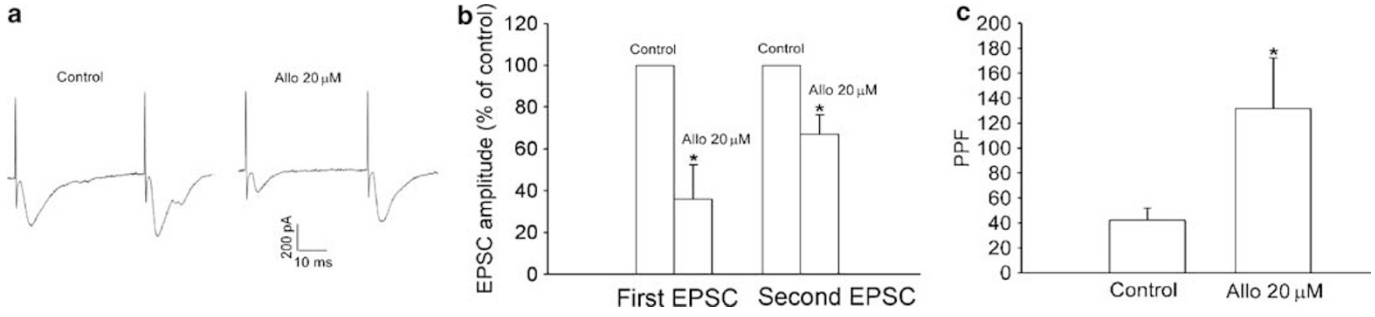

Figure 4 Effect of allopregnanolone (Allo) on PPF in the pyramidal cells of the layer V–VI of the medial prefrontal cortex. (a) Representative traces of PPF before and during application of 20 μM Allo. Holding potential is −70 mV. (b) The degree to which 20 μM Allo reduced the amplitude of the first and second EPSC. $n = 5$, $^*P < 0.05$, compared to control (c) The averaged PPF before and during application of 20 μM Allo. $n = 5$, $^*P < 0.05$, compared to control.

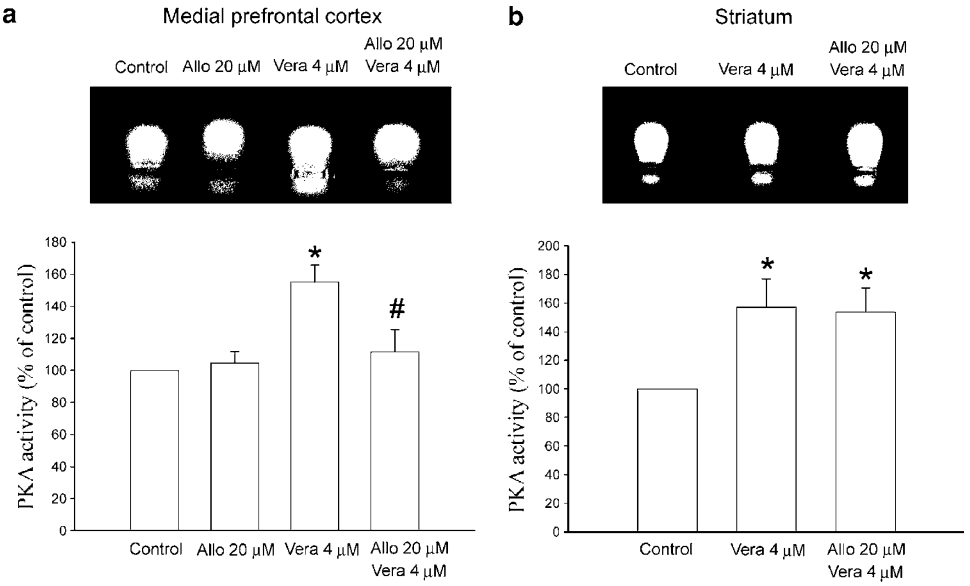

Figure 5 Effect of allopregnanolone (Allo) on the activity of PKA with and without veratridine (Vera). Top figure in (a, b): representative gel electrophoresis for PKA activity assay. Bottom figure in (a, b): the averaged results in a group of cells before and after Allo. (a) Effect of Allo (20 μM) on the activity of PKA with and without Vera (4 μM) in synaptosomes of the medial prefrontal cortex. $n=4$, $^{*}P<0.05$, compared to control group, $^{\#}P<0.05$, compared to Vera group. (b) Effect of Allo (20 μM) on the activity of PKA with and without Vera (4 μM) in synaptosomes of the striatum. $n=5$, $^{*}P<0.05$, compared to control group.

the residual PKA activity in the presence of verapamil was reduced to $2.9\pm0.4\%$ compared with verapamil alone group ($n=4$), which was significantly different from the inhibition produce by allopregnanolone alone ($30.5\pm5.3\%$, $n=4$, $P<0.05$). We also checked the influence of verapamil on the allopregnanolone-mediated inhibition of the veratridine-evoked increase in the frequency of sEPSCs and obtained a similar result to that of the PKA experiment ($n=6$, Figure 7b). These results suggest that the allopregnanolone-mediated inhibition of the high K^+-evoked PKA activation may be through the inhibition of L-type calcium channel. To confirm this statement, we observed the influence of another L-type calcium channel antagonist nimodipine on the effect of allopregnanolone. The result was consistent with that of verapamil experiment. Nimodipine (10 μM) caused a suppression of the high K^+-evoked increase in PKA activity by $25.7\pm4.2\%$ (from 214.1 ± 14.7 to $158.7\pm12.1\%$ of control, $n=4$, $P<0.05$) and completely blocked the effect of allopregnanolone (Figure 7c, $n=4$). The averaged allopregnanolone (20 μM)-induced decrease in the residual PKA activity in the presence of nimodipine was reduced to $-1.0\pm2.6\%$ compared with nimodipine alone group ($n=4$), which was significantly different from the inhibition produced by allopregnanolone alone ($30.5\pm5.3\%$, $n=4$, $P<0.05$).

We next examined whether the inhibition of N- and P/Q-type calcium channels contributes to the allopregnanolone-mediated inhibition of the high K^+-evoked PKA activation. As shown in Figure 7d and 7e, application of the N-type calcium channel antagonist ω-conotoxin (0.5 μM) and the P/Q-type calcium channel antagonist ω-agatoxin (0.5 μM) reduced the high K^+-evoked PKA activation by $18.3\pm5.7\%$ (from 189.7 ± 37.1 to $148.8\pm18.2\%$ of control, $n=4$, $P<0.05$) and by $19.3\pm6.3\%$ (from 178.5 ± 13.4 to $142.2\pm9.1\%$ of control, $n=4$, $P<0.05$), respectively, and after the application of ω-conotoxin and ω-agatoxin allopregnanolone was still able to inhibit the residual high K^+-evoked PKA activation by $19.8\pm4.4\%$ ($n=4$, $P<0.05$, Figure 7d) and $26.0\pm5.8\%$ ($n=4$, $P<0.05$, Figure 7e), respectively, which was not statistically different from the inhibition produced by allopregnanolone alone ($30.5\pm5.3\%$, $n=4$, $P>0.05$).

Allopregnanolone Inhibits L-Type Calcium Channel Agonist-Evoked Increase in the PKA Activity, Intrasynaptosomal Calcium Concentration and Frequency of sEPSCs

To further demonstrate an inhibitory effect of allopregnanolone on L-type calcium channel activation-evoked PKA activation, we observed the effect of allopregnanolone on the L-type calcium channel agonist Bay k8644-evoked increase in the PKA activity. The result showed that Bay k8644 (1 μM) alone could significantly increase the PKA activity and the averaged increasing percentage of the PKA activity after Bay k8644 was $152.0\pm8.3\%$ of control ($n=5$, $P<0.05$, Figure 8a), but in the presence of allopregnanolone (20 μM) the effect of Bay k8644 was canceled (Figure 8a). We also examined the effect of allopregnanolone on the Bay k8644-evoked increase in the synaptosomal calcium concentration in the presence of picrotoxin (200 μM) and higher concentration of ryanodine (10 μM) to block $GABA_A$ receptors and endoplasmic reticulum calcium release, respectively. As shown in Figure 8b, an addition of Bay k8644 (1 μM) resulted in a significant increase in intrasynaptosomal Ca^{2+} concentration from 307.0 ± 10.6 nM to 410.0 ± 19.2 at 5 min after Bay k8644 ($n=4$, $P<0.05$), whereas in the control conditions there were not significant changes at the same time point ($n=4$, $P>0.05$, Figure 8b). However, in the presence of allopregnanolone (20 μM) the

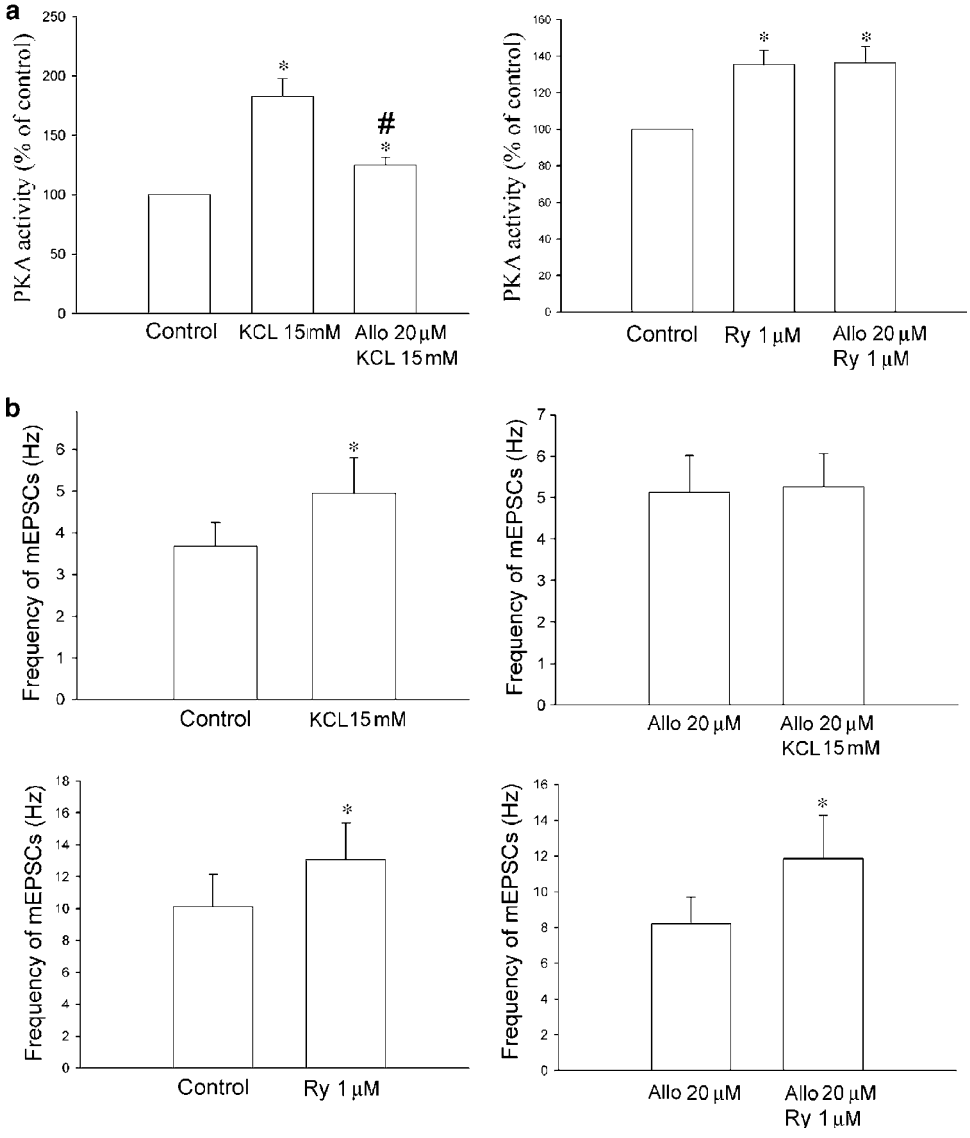

Figure 6 Effect of allopregnanolone (Allo) on extracellular calcium influx- and intracellular calcium store release-evoked PKA activation and the increase in the frequency of sEPSCs. (a) Left panel: effect of Allo (20 μM) on the high K^+ (15 mM)-evoked increase in the PKA activity in synaptosomes of the medial prefrontal cortex. $n=4$, $^*P<0.05$, compared to control group, $^{\#}P<0.05$, compared to high K^+ group. Right panel: effect of Allo (20 μM) on the ryanodine (Ry, 1 μM)-evoked increase in the PKA activity in synaptosomes of the medial prefrontal cortex. $n=4$, $^*P<0.05$, compared to control group. (b) Top panels: effect of Allo (20 μM) on the high K^+ (15 mM)-evoked increase in the frequency of sEPSCs in the pyramidal cells of the layers V–VI of the medial prefrontal cortex. $n=6$, $^*P<0.05$, compared to control group. Bottom panels: effect of Allo (20 μM) on the Ry (1 μM)-evoked increase in the frequency of sEPSCs in the pyramidal cells of the layers V–VI of the medial prefrontal cortex. $n=5$, $^*P<0.05$, compared to control group or Allo group.

increasing effect of Bay k8644 on the Ca^{2+} concentration disappeared ($n=4$, $P>0.05$, Figure 8b). In addition, we observed the influence of allopregnanolone on the Bay k8644-evoked increase in the frequency of sEPSCs and obtained a similar result to those of the PKA and intrasynaptosomal calcium experiments (Figure 8c, $n=7$). Moreover, here we again observed the effect of lower concentration of allopregnanolone on the L-type calcium channel agonist-evoked increase in the PKA activity and intrasynaptosomal calcium concentration. The result showed that allopregnanolone at lower concentration of 0.1 μM could also significantly inhibit the Bay k8644 (1 μM)-evoked increase in the PKA activity and intrasynaptosomal calcium concentration (Figure 9a and b, $n=4$). However, the extent of the inhibition evoked by 0.1 μM allopregnanolone was weaker than that evoked by 20 μM allopregnanolone. We also observed the effect of lower concentration of allopregnanolone on PPF. The result showed that after the addition of 0.1 μM allopregnanolone, the first EPSC was inhibited by $33.0\pm7.1\%$, but the second EPSC only by $24.6\pm5.8\%$ (Figure 9c, $n=6$). Therefore, PPF was increased after allopregnanolone. The averaged PPF increased from 51.0 ± 11.6 before to $75.8\pm14.4\%$ at 10 min after allopregnanolone (Figure 9d, $n=6$, $P<0.05$).

DISCUSSION

The present finding that allopregnanolone has no effects on spontaneous presynaptic glutamate release, but significantly

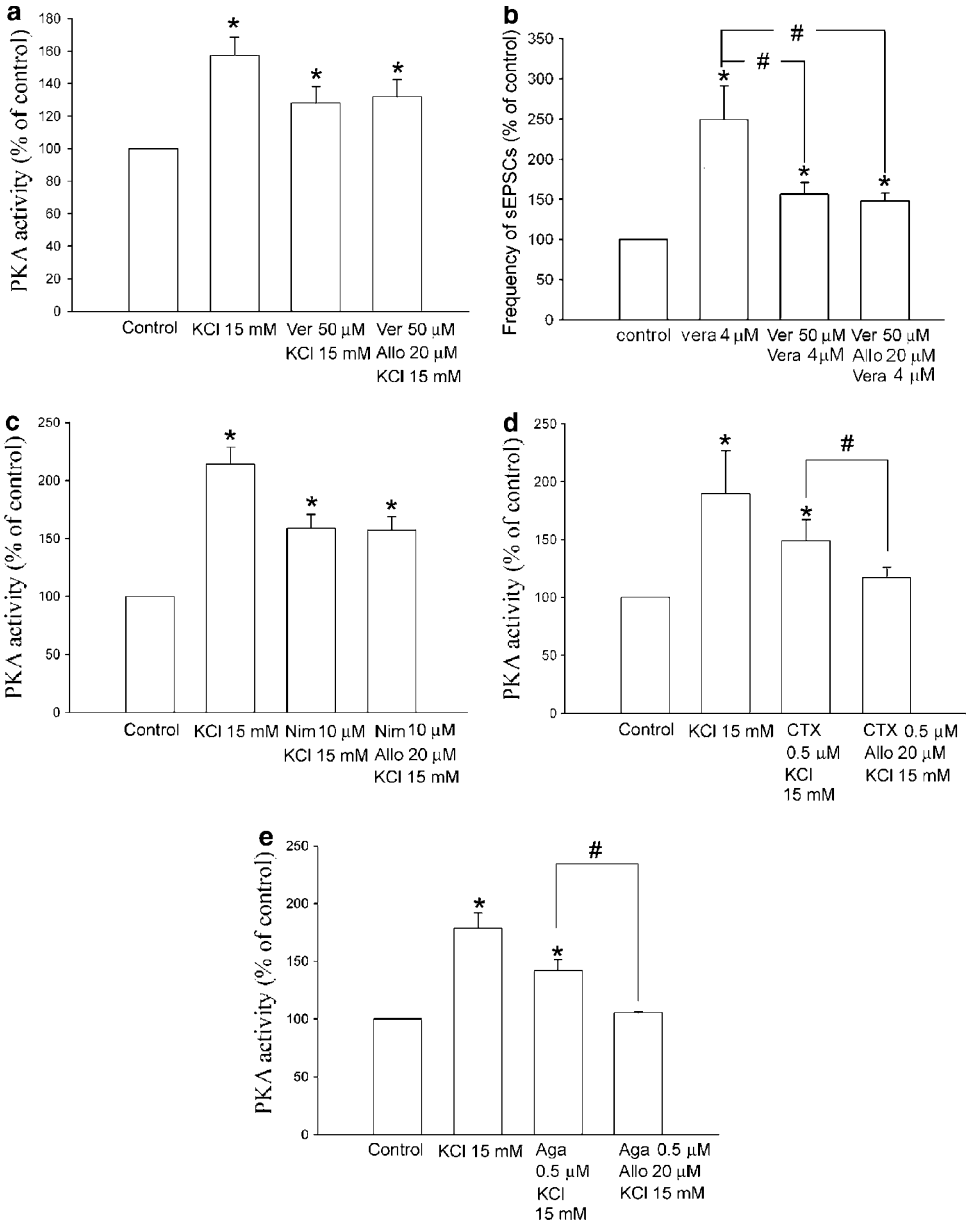

Figure 7 The influence of calcium channel subtype-selective antagonists on the effect of allopregnanolone (Allo). (a) The influence of the L-type calcium channel antagonist verapamil (Ver, 50 μM) on the effect of Allo (20 μM) on the high K^+ (15 mM)-evoked PKA activation in synaptosomes of the medial prefrontal cortex. $n=4$, $^*P<0.05$, compared to control group. (b) The influence of the L-type calcium channel antagonist verapamil (Ver, 50 μM) on the effect of Allo (20 μM) on the veratridine (Vera, 4 μM)-evoked increase in the frequency of sEPSCs in the pyramidal cells of the layers V–VI of the medial prefrontal cortex. $n=6$, $^*P<0.05$, compared to control group, $^{\#}P<0.05$, compared to Vera group. (c) The influence of the L-type calcium channel antagonist nimodipine (Nim, 10 μM) on the effect of Allo (20 μM). $n=4$, $^*P<0.05$, compared to control group. (d) The influence of the N-type calcium channel antagonist ω-conotoxin (CTX, 0.5 μM) on the effect of Allo (20 μM). $n=4$, $^*P<0.05$, compared to control group, $^{\#}P<0.05$, compared to CTX group. (e) The influence of the P/Q-type calcium channel antagonist ω-agatoxin (Aga, 0.5 μM) on the effect of Allo (20 μM). $n=4$, $^*P<0.05$, compared to control group, $^{\#}P<0.05$, compared to Aga group.

inhibits the depolarizing agent and electrical stimulus evoked presynaptic glutamate release in the medial prefrontal cortex is interesting because it shows that the effect of allopregnanolone on presynaptic glutamate release is neuronal activity-dependent, that is, when presynaptic terminals are under an unstimulated state, allopregnanolone has no effects, but if the terminals are stimulated, allopregnanolone has an inhibitory effect on their glutamate release. This statement was consistent with the result by Tauboll *et al* (1993) who showed that allopregnanolone reduced high K+-induced glutamate release from identified nerve terminals in rat hippocampus by a semiquantitative immunocytochemical approach, although they did not observe the effect of allopregnanolone on the glutamate release under resting state. In addition, our study also showed that this effect of allopregnanolone appeared to have some extent of selectivity for brain regions because our results showed that allopregnanolone had the effect in the medial prefrontal cortex, but not in the striatum.

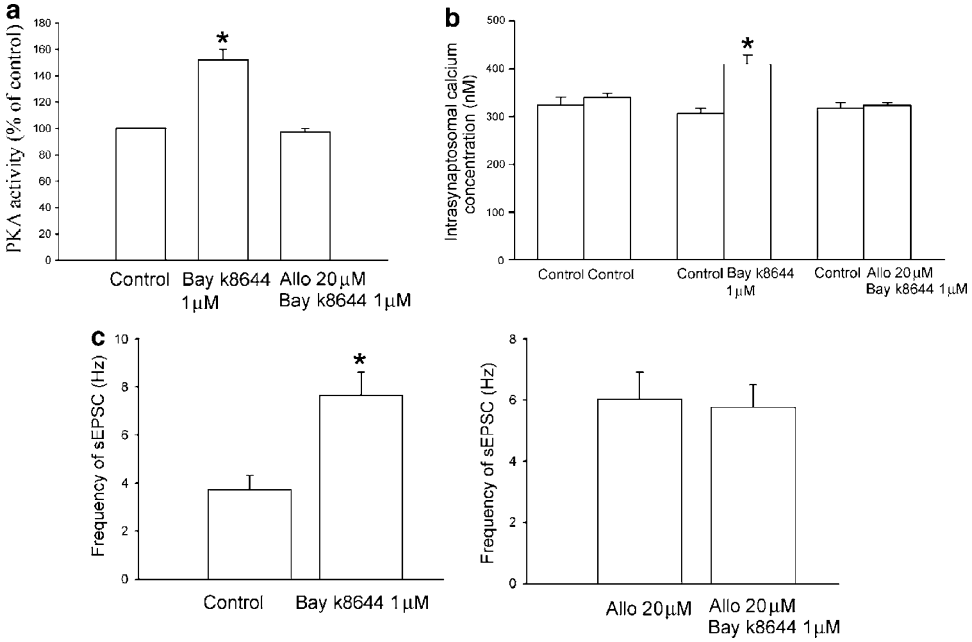

Figure 8 Effect of allopregnanolone (Allo) on the L-type calcium channel agonist-evoked increase in the PKA activity, intrasynaptosomal calcium concentration and frequency of sEPSCs. (a) Effect of Allo on the L-type calcium channel agonist Bay k8644 (1 μM) -evoked increase in the PKA activity in synaptosomes of the medial prefrontal cortex. $n=5$, $^{*}P<0.05$, compared to control group. (b) Effect of Allo (20 μM) on the L-type calcium channel agonist Bay k8644 (1 μM)-evoked increase in the intrasynaptosomal calcium concentration in synaptosomes of the medial prefrontal cortex. $n=4$, $^{*}P<0.05$, compared to control before Bay k8644. (c) Effect of Allo on the L-type calcium channel agonist Bay k8644 (1 μM) -evoked increase in the frequency of sEPSCs in the pyramidal cells of the layers V–VI of the medial prefrontal cortex. $n=7$, $^{*}P<0.05$, compared to control group.

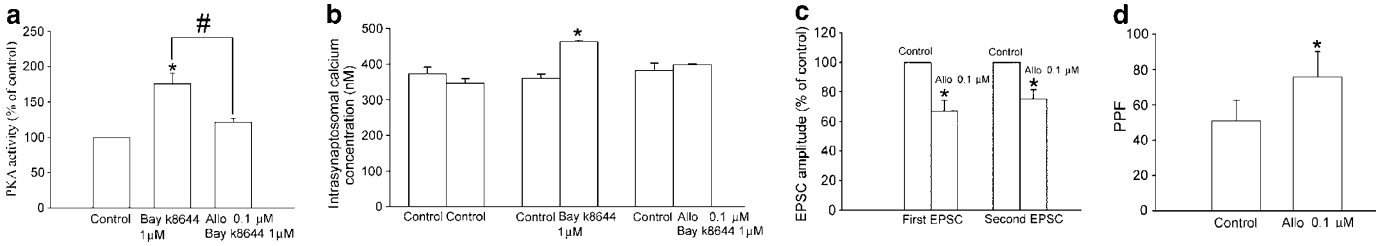

Figure 9 Effect of lower concentration of allopregnanolone (Allo) on the L-type calcium channel agonist-evoked increase in the PKA activity and intrasynaptosomal calcium concentration. (a) Effect of Allo (0.1 μM) on the L-type calcium channel agonist Bay k8644 (1 μM) -evoked increase in the PKA activity in synaptosomes of the medial prefrontal cortex. $n=4$, $^{*}P<0.05$, compared to control group, $^{\#}P<0.05$, compared to Bay k8644 group. (b) Effect of Allo (0.1 μM) on the L-type calcium channel agonist Bay k8644 (1 μM)-evoked increase in the intrasynaptosomal calcium concentration in synaptosomes of the medial prefrontal cortex. $n=4$, $^{*}P<0.05$, compared to control before Bay k8644. (c) The degree to which 0.1 μM Allo reduced the amplitude of the first and second EPSC. $n=6$, $^{*}P<0.05$, compared to control. (d) The averaged PPF before and during application of 0.1 μM Allo. $n=6$, $^{*}P<0.05$, compared to control.

Presynaptic evoked glutamate release is a rather complicated process involving activation of a series of presynaptic proteins, such as ion channels and enzymes etc. Among them, PKA activation is an important upstream event of evoked glutamate release (Leenders and Sheng, 2005; Millan *et al*, 2003; Grilli *et al*, 2004). Therefore, to explore the mechanism for the inhibitory effect of allopregnanolone on evoked glutamate release, here we first observed the effect of allopregnanolone on the depolarizing agent-evoked PKA activation. The result showed that allopregnanolone significantly inhibited the depolarizing agent-evoked PKA activation. This result is interesting for two reasons. First, this inhibitory effect might be an important mechanism for the inhibitory effect of allopregnanolone on evoked presynaptic glutamate release because, in addition to the literatures that already showed the importance of PKA activation in evoked glutamate release, our experiment also showed that if mimicking the inhibitory effect of allopregnanolone on PKA activity with H89, the depolarizing agent-evoked increase in the frequency of sEPSCs was significantly inhibited. Second, we can use this effect as a measure to further study the possible upstream mechanism of the PKA inhibition by allopregnanolone.

We first eliminated the possibility of a direct inhibitory effect of allopregnanolone on the activated PKA because our result showed that allopregnanolone had no effects on the PKA activator forskolin-evoked activation of PKA. This was also supported by our electrophysiological result that showed that allopregnanolone had no effects on the PKA

activator forskolin-evoked increase in the frequency of sEPSCs.

The stimulus we used here to evoke glutamate release was veratridine, high K^+ and electrical pulses. Although there are some differences among them in the mechanism to evoke glutamate release, one common action of them is that they all promote extracellular calcium influx. Thus, one possible mechanism for the inhibitory effect of allopregnanolone on the PKA activation is that allopregnanolone may have an inhibitory effect on extracellular calcium influx-evoked PKA activation. To test this hypothesis, we used high K^+, which was only calcium channel-dependent (Sitges and Galindo, 2005; Lingamaneni and Hemmings, 1999), to induce the extracellular calcium influx-evoked PKA activation and then observed the effect of allopregnanolone on high K^+-evoked PKA activation. The result showed that allopregnanolone could significantly inhibit the high K^+-evoked PKA activation. Interestingly, allopregnanolone had no influence on the intracellular calcium store release-evoked PKA activation. This statement was further supported by our electrophysiological result that showed that allopregnanolone had no effects on the intracellular calcium store release-evoked increase in the frequency of sEPSCs. These evidences suggest that the target site for the effect of allopregnanolone on the PKA activation may not be at downstream of intracellular calcium, but at the level of membrane calcium channels.

It has been known that high K^+ can open L-, N-, and P/Q-type calcium channels (Lopez *et al*, 2001; Dunlap *et al*, 1995). Thus, the inhibitory action of allopregnanolone on the high K^+-evoked PKA activation may result from the inhibition of the activity of some of these different calcium channel subtypes. In this aspect, our results suggested that the allopregnanolone-mediated inhibition of the high K^+-evoked PKA activation was most probably through the inhibition of L-type calcium channels, but not through N- and P/Q-type calcium channels because L-type calcium channel antagonists, but not the N- and P/Q-type calcium channel antagonist, blocked the effect of allopregnanolone. In addition, our results that allopregnanolone inhibited the L-type calcium channel agonist-evoked increase in the PKA activity, intrasynaptosomal calcium concentration and frequency of sEPSCs further supported this conclusion.

Another interesting phenomenon we observed here was that allopregnanolone could inhibit both the depolarizing agent-evoked PKA activation and the increase in the frequency of sEPSCs in the medial prefrontal cortex, but not in the striatum. Although the cell type recorded here in these two brain regions is different (the pyramidal cells in the medial prefrontal cortex are glutamatergic cell that releases glutamate as its neurotransmitter, while the spiny cells in the striatum are GABAergic that releases GABA as its neurotransmitter), both these two types of cells receive glutamatergic inputs from other brain regions. Thus, the present finding indicates that for these two types of cells allopregnanolone selectively modulate glutamatergic inputs in the pyramidal cells of the medial prefrontal cortex. The reasons for this difference remained unknown. One possible reason is that there may be a different role of L-type calcium channels in mediating presynaptic glutamate release in the medial prefrontal cortex and the striatum, so the consequence of the inhibition of L-type calcium channels by allopregnanolone on presynaptic glutamate release is different in these two brain regions. This speculation was supported by the observations that L-type calcium channels had little role in the mediation of presynaptic glutamate release in the striatum (Lovinger *et al*, 1994; Bargas *et al*, 1998), but contributed much to the presynaptic glutamate release in the medial prefrontal cortex (Lopez *et al*, 2001). In addition, our own results that the L-type calcium channel antagonist verapamil could significantly inhibit the veratridine-evoked PKA activation in the medial prefrontal cortex, but not in the striatum also were consistent with these observations.

L-type calcium channels have been found to regulate a multitude of neuronal processes including neurotransmitter release, gene expression, mRNA stability, neuronal survival, ischemic-induced axonal injury, synaptic efficacy, and the activity of other ion channels (Lipscombe *et al*, 2004), but it appears that there may be a different role of L-type calcium channels in different brain regions, especially for the effect on neurotransmitter release (Lipscombe *et al*, 2004; Li and Bennett, 2003; Wiser *et al*, 1999; Sabria *et al*, 1995; Reuter, 1996; Lopez *et al*, 2001; Bargas *et al*, 1998). In addition, it was reported that some pathophysiologic stimulus such as stress and psychostimulants could significantly elevate the expression of L-type calcium channels in the cerebral cortex (ntkiewicz-Michaluk *et al*, 1990, 1993, 1994a, b, 1995; Mamczarz *et al*, 1994, 1999) and this elevation had been proposed to be involved in etiology of a variety of psychiatric disorders such as schizophrenia, morphine abstinence, and neuroleptic withdrawal (ntkiewicz-Michaluk *et al*, 1994a, b, 1997, 1995, 1993; Mamczarz *et al*, 1994, 1999; ntkiewicz-Michaluk, 1999). Moreover, the glutamate release in the medial prefrontal cortex is also important in etiology of a variety of psychiatric disorders (Adams and Moghaddam, 1998; Moghaddam *et al*, 1997; Moghaddam and Adams, 1998). Therefore, the present finding that allopregnanolone inhibits the L-type calcium channel activation-evoked glutamate release in the medial prefrontal cortex is of important significance for understanding the possible antipsychotic effect of allopregnanolone.

The concentrations of allopregnanolone used in the present study (0.1–20 μM) appeared to be higher than that expected for a physiological relevant conditions of about 4 ng/g of rat brain tissue (Marx *et al*, 2000). However, the present known concentrations of allopregnanolone were determined in homogenates of brain tissue, and the actual synaptic concentrations of allopregnanolone were unknown. It is entirely possible that allopregnanolone reaches micromolar levels in synaptic regions under certain physiological conditions. In addition, even for the typical effect of allopregnanolone on GABAergic neurotransmission in the cortex, the effective concentration range of allopregnanolone in most studies was also above 0.1 μM (Wilson and Biscardi, 1997; Puia *et al*, 2003). Therefore, our results, in addition to pharmacological significance, may be physiologically relevant.

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