Novel Mechanism to Explain Ketamine Efficacy Against Nerve Agent-Induced Seizures

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ABSTRACT TITLE: NOVEL MECHANISM TO EXPLAIN KETAMINE EFFICACY AGAINST NERVE AGENT-INDUCED SEIZURES

Nerve agent (NA) toxicity results from increased cholinergic activity both peripherally in muscle and centrally in the brain. Intoxication with NAs produces symptoms of hypercholinergic activity, including tremors, salivation, bradycardia, muscle paralysis, seizures, respiratory failure, and death. Current countermeasures in the U.S. consist of the peripherally acting pretreatment pyridostigmine bromide, a reversible cholinesterase (ChE) inhibitor, followed by the muscarinic receptor antagonist atropine and an oxime to reactivate ChE. In addition, benzodiazepines are necessary to halt the convulsions that arise from NA intoxication in the central nervous system (CNS). While compounds are continually being evaluated as newer generation anticonvulsants and neuroprotectants against NA intoxication, ketamine (KET) is an FDA-approved drug that provides seizure protection against NA challenge. KET, a dissociative anesthetic commonly used in veterinary medicine and pediatrics, is an analog of the psychotomimetic phencyclidine (PCP). KET prevents lethality and halts seizures when administered 30 min after NA challenge followed by atropine. Using the whole-cell patch-clamp technique to investigate the interactions of these compounds with native neuronal α7 nAChRs present in rat hippocampal neurons in culture, we provide evidence that both KET and PCP are potent inhibitors of central nicotinic acetylcholine receptors (nAChRs), presynaptic switches that control release of excitatory neurotransmitters. The behaviorally inactive KET analog piperidinylcyclohexane carbonitrile (PCC) did not inhibit α7 nAChRs at high concentrations. Agonists were delivered via a U-tube, whereas antagonists were applied in admixture with the agonists and via the background perfusion. KET and PCP provide neuroprotection through a powerful antagonism of excitatory neurotransmission, namely, pre-synaptic inhibition of glutamate release and post-synaptic inhibition of N-methyl-D-aspartate (NMDA)-type glutamate receptors. KET or their analogs may represent a new generation of centrally active post-exposure therapeutics against NA challenge. Timely provision of safe post-exposure treatment countermeasures to

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treat intoxication with NA is essential to preserve and sustain combat effectiveness of the warfighter. These post-exposure treatments represent an improvement upon the current pretreatment strategy and may expand upon the current military, first responder, and hospital pharmaceutical cache to treat casualties of NA exposure.

1.0 INTRODUCTION

Organophosphorus (OP) compounds have long been used as pesticides and chemical warfare agents. Occupational exposure to OP pesticides afflicts millions of people and leads to hundreds of thousands of deaths every year throughout the world.[1] OPs used as weapons of mass destruction are far more toxic than those used as pesticides and are suitable for terrorist attacks such as those that took place in Japan in 1994 and 1995.[2,3] It appears that thousands of tons of these chemical warfare agents are still available globally.[4] Intoxication with OPs produces typical signs and symptoms of hypercholinergic activity, including tremors, salivation, hypotension, bradycardia, convulsions, cyanosis, and death, primarily due to respiratory failure secondary to bronchoconstriction, excessive airway secretion, paralysis of essential muscles of respiration, and inhibition of respiratory control centers in the brain.[5] These symptoms arise primarily from the irreversible inhibition of cholinesterases (ChE), enzymes involved in the metabolism of the neurotransmitter acetylcholine (ACh). Thus, treatment of acute OP poisoning includes, in addition to supportive management of compromised airways with endotracheal intubation and mechanical ventilation, atropine and oxime reactivators. The application of atropine is used to block excessive activation of muscarinic receptors, while oximes such as pralidoxime (2-PAM) are needed to reactivate ChE. NA-induced seizures, if left unchecked, will progress to status epilepticus and contribute to profound brain damage.[6,7,8] Anticonvulsants are therefore required for clinical management.

The current FDA-approved nerve agent pretreatment, pyridostigmine bromide (PB), is able to increase survival but cannot prevent seizures or other centrally mediated toxic actions of NA exposure. The highly lipid soluble nerve agents, such as soman, sarin, and VX, easily penetrate the blood brain barrier (BBB), irreversibly inhibit brain ChE and cause seizures. PB, on the other hand, does not cross the BBB, and additional therapeutic treatment with benzodiazepine (BZD) anticonvulsants is required. Racemic ketamine (KET), a general anesthetic used in pediatric and veterinary medicine, has been shown to be very effective with atropine sulfate in stopping seizures and protecting sensitive brain areas when given up to 1 h after a lethal challenge of soman.[9] KET, a phencyclidine derivative, is thought to exert most of its activity via antagonism of NMDARs in the CNS. However, this action alone does not explain why KET demonstrates efficacy to stop seizures while other NMDAR antagonists do not.

KET has an interesting pharmacology. It is a widely used anesthetic in pediatric and veterinary medicine; however, in adults, it can cause hallucinatory emergence reactions. It is widely held that general anesthetics act on one or more members of the superfamily of ligand-gated ion channels that include $GABA_A$, NMDARs, nAChRs, $5\text{-}HT_3$Rs, and glycine receptors. $GABA_A$ and glycine receptors, on the other hand, are very insensitive to KET.[10] The majority of KET action has been attributed through antagonism of NMDA-type glutamate receptors. KET was discovered to act as an NMDAR antagonist on Renshaw cells of the cat spinal cord[11] and rat cortex.[12] KET blocks NMDARs through a use-dependent, open channel blocking mechanism and via allosteric inhibition.[13] KET actions on the neuronal nAChR system have largely been ignored, despite their inhibition of muscle-type nAChRs. KET has been shown to inhibit the response to ACh or nicotine in muscle[14] and α4-containing nAChRs.[15]

The whole-cell mode of the patch-clamp technique was used to investigate the interaction of KET and PCP with native, neuronal α7 nAChRs expressed in cultured neurons from rat hippocampus. Agonists were

delivered via a fast U-tube system of drug application and removal, while PCP and KET were applied as an admixture with their agonist and via the background perfusion. We hypothesize that the halt of seizures by KET antagonism of glutamatergic neurotransmission in the CNS is accomplished by 1) post-synaptic NMDAR blockade and 2) presynaptic blockade of glutamate, the predominant excitatory neurotransmitter in the CNS, via α7 nAChR blockade.

2.0 MATERIALS AND METHODS

2.1 Neuronal Cultures from Rat Hippocampus

Primary fetal cultures from fetal rat hippocampus were purchased as pelleted cells (Neuropure™, San Diego, CA). The cells were plated out onto 35-mm culture dishes and placed in an incubator at 37°C and 5% CO_2. Pelleted neurons dissected from the hippocampus of fetal rat pups were purchased from Genlantis (San Diego, CA). These cultured neurons were washed three times in a modified Eagle's medium (MEM, GIBCO) to halt degradative enzyme activity, and the medium was replaced with fresh MEM10/10 containing fetal bovine serum (FBS, 10%), heat-inactivated horse serum (HS, 10%), glutamine (2 mM), and DNase (40 ug/ml). The neurons in suspection were dissociated with the aid of a Pasteur pipette. About 700,000 dissociated cells suspended in 2 ml of MEM10/10 were plated on collagen-precoated Petri dishes (Nunc 35-mm diameter) and incubated in a humid atmosphere of 10% CO2/90% air at 35 C. The MEM10/10 was replaced 24 hours later with MEM10, a MEM enriched with HS (10%) and glutamine (2 mM). After 7 days, an admixture of 5-fluoro-2'-deoxyuridine/uridine (FDUR) was added to the culture medium to inhibit glial cell growth. Twenty-four hours later, the culture medium was replaced with fresh MEM10. Twice a week thereafter, the medium was replaced by fresh MEM10. Electrophysiological experiments were performed on hippocampal neurons cultured for 7-35 days.

2.2 Electrophysiological Recordings

The culture dishes containing the dissociated neurons were placed on the stage of an inverted Zeiss microscope (Carl Zeiss MicroImaging, Inc., West Germany) and visualized with phase-contrast optics prior to patch clamp electrophysiology. The whole-cell mode of the patch clamp configuration was used to record whole-cell and cell-attached current recordings on 15- to 30-day-old cultured hippocampal neurons from fetal rat pups, according to standard patch clamp techniques[16] using an EPC-7 PLUS patch clamp amplifier (HEKA Instruments Inc., Southboro, MA). Signals were filtered at 1-2 kHz and either stored in a VCR for later analysis or directly sampled with a PC using the PCLAMP9.1 program (Axon Instruments, Foster City, CA). The composition of the external solution used to bathe the cultured neurons was (in mM) NaCl 165, KCl 5, $CaCl_2.2H_2O$ 1, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) 5, dextrose 10 (pH was adjusted to 7.3 with NaOH; Osm: 340 mOsm). In all experiments, atropine (1 μM) and tetrodotoxin (TTX, 200 nM) were added to the external solution to block muscarinic receptors and voltage-gated Na^+ channels, respectively. The internal solution for recordings from cultured neurons had the following composition (in mM): CsCl 80, CsF 80, ethyleneglycoltetraacetic acid (EGTA) 10, CsOH 22.5 and HEPES 10 (pH was adjusted to 7.3 with CsOH; 340 mOsm). When whole-cell currents were evoked by activation of α7 nAChRs, rundown was minimized by adding the following ATP-regenerating compounds to the internal solution: tris-phosphocreatine 20.0 mM, tris-ATP 5.0 mM and creatine-phosphokinase 50 Units/ml. As a result, the concentrations of CsCl and CsF in the internal solution were subsequently decreased to 60 mM so that the osmolarity of the ATP-regenerating internal solution was maintained at approximately 340 mOsm. When filled with the internal solutions described above, the recording pipettes had resistances of 2-5 MΩ for cultured neurons. The access resistance was ~15 MΩ and was not compensated for during experiments. Results were not used when the access resistance changed significantly during the course of data acquisition.

2.3 Patch Pipettes

Microelectrode patch recording pipettes were pulled from borosilicate glass from a Model P-97 Flaming/Brown Micropipette Puller to have a resistance between 2-5 MΩ for the electrophysiological recordings. Positive pressure was applied to the internal solution-containing microelectrode, which was positioned over the cell with the aid of a Narishige mechanical micromanipulator, allowing the formation of a GΩ seal between the pipette tip and the cell membrane.

2.4 Drug Application

Whole-cell currents were induced by fast application of the agonist (NMDA, ACh or choline, a selective α7 nAChR agonist) to the neurons using a U-shaped glass tube[17] positioned 50 μM above the cell. In all experiments, antagonists were delivered to the neurons via the U-tube (as admixtures with agonists) and via the bath perfusion.

The agonist-containing solutions were applied to the cells through a hole (250 to 300 μM diameter) made at the apex of the U-tube. The U-tube design for agonist application is a unique design for fast delivery and removal of agonist applied to neurons. This is necessary to be able to record the fast desensitizing currents characteristic of α7 nAChR activation. KET (and analogs) were applied to the external solution, bathing the neurons as well as an admixture in the U-tube with the agonist.

2.5 Data Analysis

Peak amplitude, rise time, and decay-time constants (□decay) of whole-cell currents were analyzed using the PCLAMP9.1 program. Rundown of whole-cell currents triggered by activation of α7 nAChRs was corrected according to the following analysis. Agonist pulses were applied to the neurons at 2-min intervals for as long as needed to stabilize the current amplitudes before exposure of the neurons to agonist-antagonist admixtures. After full reversibility of the effects of antagonists was achieved upon washing of the neurons, agonist pulses were applied to the neurons at 2-min intervals for an additional 4-15 min. Plots of the amplitudes of agonist-evoked currents versus recording time were fit by either linear regression or first order exponential decay of the points obtained before exposure of the neurons to the antagonists and after wash. The expected amplitudes of agonist-evoked currents at any given time were subsequently estimated. Concentration-response curves were fit by the Hill equation: $I = (I_{max} \times [A]^{nH}) / ([A]^{nH} + EC_{50}{}^{nH})$, where I and I_{max} are the measured and the maximum current amplitudes, respectively, [A] is the agonist concentration, n_H is the Hill coefficient and EC_{50} is the agonist concentration producing half-maximum response. Data are presented as mean ± S.E.M.

3.0 RESULTS

In the presence of atropine (1 μM) and TTX (200 nM), application of the α7-selective agonist choline (10 mM) to cultured hippocampal neurons evoked whole-cell currents decaying to baseline before the end of the agonist pulse. These α7 nAChR-mediated currents, which are referred to as type IA currents, were the result of activation of α7 nAChRs present in the soma and proximal dendrites of hippocampal neurons from which recordings were obtained. These fast desensitizing, choline-activated currents were reversibly blocked by the α7 nAChR-selective competitive antagonist methyllycaconitine (MLA, 1 nM), but not by the α4β2 nAChR competitive antagonist dihydro-β-erythroidine (DHβE, 200 nM) (Fig. 1). The natural neurotransmitter ACh can be used to elicit type IA α7 nAChR currents, but it is non-specific and often elicits an α4β2 nAChR

component. These are non-selective currents called type IB currents. Therefore, the α7-selective agonist choline was used in this study to elicit pure α7 nAChRs.

3.1 KET inhibits somatodendritic α7 nAChRs in cultured hippocampal neurons

The peak current of choline (10 mM)-evoked type IA currents was reduced after perfusion of cultured hippocampal neurons with external solution containing KET (10 μM -300 μM) (Fig. 2A). At the lowest test concentration, *i.e.*, 10 μM, KET decreased by approximately 20% the amplitude of type IA currents. On average, it took 2 min to observe the onset of the inhibitory effect of KET on α7 nAChRs in cultured hippocampal neurons; the time required to achieve final inhibition for each concentration of KET tested was 6-8 minutes. In addition, inhibition of α7 nAChRs by KET was fully reversible upon washing of the neurons with KET-free external solution for ~ 8-10 min (Fig. 2A). The onset for reversal during the washout period was seen almost immediately in the first pulse after washout, but complete reversibility was achieved only after an 8- to 10-minute washout.

The inhibitory effect of KET on α7 nAChRs was also concentration dependent. Fitting the concentration-response relationship by the Hill equation revealed that KET blocks α7 nAChRs with an IC_{50} of 40.0 ± 3.1 μM (Fig. 2B) and an n_H of 0.95 ± 0.05. At 300 μM, KET reduced by 85% the amplitude of type IA currents.

The decay phase of type IA currents evoked by choline in the absence and in the presence of KET was best fit by a single exponential. The τ–decay of type IA currents evoked by choline (10 mM) was 19.1 ± 1.4 ms (n = 9 neurons). At concentrations ranging from 10 to 100 μM, KET had no effect on the decay phase of the currents. In the presence of KET (10-100 μM), τ-decay of choline-evoked type IA currents was 19.3 ± 1.6 ms (n = 9 neurons). At KET concentrations >100 μM, the significant reduction of the peak current amplitude made it difficult to adequately determine the decay-time constant of the currents.

3.2 Mechanism of action of KET on α7 nAChRs

To investigate the nature of the blockade of α7 nAChRs by KET, choline-evoked type IA currents were recorded before and after perfusion of the neurons with external solution containing KET (50 μM). Prior to exposure of the neurons to KET, increasing concentrations of choline (100 μM – 30 mM) evoked type IA currents with progressively larger amplitudes; the maximal response occurred at 30 mM choline. After perfusion with KET (50 μM), the maximal effect of choline was reduced. KET-induced inhibition of type IA currents was insurmountable by increasing concentrations of choline (Fig. 3A). Therefore, the interaction of KET with α7 nAChRs is non-competitive with respect to the agonist (see double reciprocal plot; Fig. 3B).

To determine the voltage dependence of KET-induced blockade of α7 nAChRs, type IA currents were evoked by choline (10 mM) in the absence or presence of KET (50 μM) at several membrane potentials. At all membrane potentials, KET reduced the amplitude of type IA currents (Fig. 4A). When normalized peak amplitude was plotted against membrane potential for currents elicted by choline and choline/KET together, the amount of inhibition produced at each membrane potential was constant (Fig. 4B). Therefore, inhibition of type IA currents by KET was voltage independent because the percent reduction of the current amplitudes by KET was not significantly different at all membrane potentials tested (Fig. 4C). These data suggest that the binding site for KET on α7 nAChRs is not within the electrical field of the membrane.

3.3 PCP inhibits somatodendritic type IA responses mediated by α7 nAChRs in culture hippocampal neurons

The peak current of choline (10 mM)-evoked type IA currents was reduced after perfusion of cultured hippocampal neurons with external solution containing PCP (1 μM - 100 μM) (Fig. 5). At the lowest test concentration, *i.e.*, 1 μM, PCP decreased by approximately 5% the amplitude of type IA currents. At 30 μM, PCP reduced by 95% the amplitude of type IA currents. On average, it took 2 min to observe the onset of the inhibitory effect of PCP on α7 nAChRs in cultured hippocampal neurons; the time required to achieve final inhibition for each concentration of PCP tested was 6-8 minutes. In addition, inhibition of α7 nAChRs by PCP was fully reversible upon washing of the neurons with PCP-free external solution for ~ 10 min (Fig. 5A). The onset for reversal during the washout period was seen almost immediately in the first pulse after washout, but complete reversibility was achieved only after an 8- to 10-minute washout.

The inhibitory effect of PCP on α7 nAChRs was concentration dependent; the higher the concentration of PCP, the larger the effect on the amplitude of type IA currents. Fitting the concentration-response relationship by the Hill equation revealed that PCP blocks α7 nAChRs with an IC_{50} of 18.1 ± 1.9 μM and an n_H of 0.99 ± 0.08 (Fig. 5B).

The decay phase of type IA currents evoked by choline in the absence and in the presence of PCP (1 μM-100 μM) was best fit by a single exponential. The □-decay of type IA currents evoked by choline (1 mM) was 27.5 ± 2.1 ms (n = 8 neurons). At concentrations ranging from 1 to 30 μM, PCP had no effect on the decay phase of the currents. In the presence of PCP (1 μM -30 μM), τ-decay of choline-evoked type IA currents was 27.8 ± 1.6 ms (n = 8 neurons). At PCP concentrations >30 μM, the reduction of the peak current amplitude was substantial, making it difficult to determine reliably the decay-time constant of the currents.

The behaviorally inactive PCP precursor 1-piperidinocyclohexanecarbonitrile (PCC) had no effect on choline-evoked type IA currents. At 30 μM, PCC did not reduce the peak current amplitude by any significant amount, while the same concentration of PCP reduces the amplitude of type IA currents by >65% (Fig. 6).

3.4 Mechanism of action of PCP on α7 nAChRs

PCP-induced inhibition of type IA currents was insurmountable by increasing concentrations of choline (data not shown). Therefore, the interaction of PCP with α7 nAChRs is also non-competitive with respect to the agonist. Also unchanged was the n_H for choline in evoking type IA currents. Inhibition of type IA currents by PCP was also voltage independent, since the percent reduction of the current amplitudes by PCP was not significantly different at all membrane potentials tested (data not shown). These data suggest that the binding site for the PCP on α7 nAChRs is also not within the electrical field of the membrane.

4.0 DISCUSSION

4.1 KET and PCP: Negative allosteric modulators of α7 nAChRs

Results obtained from the electrophysiological experiments carried out in hippocampal neurons demonstrated that KET and PCP are non-competitive antagonists of somatodendritic α7 nAChRs. Assuming a similar sensitivity and mechanism of action on presynaptic/preterminal α7 nAChRs, KET and its analogs are antagonists of presynaptic α7 nAChRs, causing inhibition of glutamate release. This concept fits well with the current hypoglutamatergic state believed to be involved in schizophrenia[18] since KET and PCP are

considered schizophrenomimetics. These data are also consistent with the role of the α7 subtype of nAChRs in the hippocampus. In this brain region, α7 is the predominant nAChR subtype and can be found presynaptically to control the release of the excitatory neurotransmitter glutamate (Fig. 7A). The combinatorial effect from direct inhibition of NMDA-type glutamate receptors and indirect inhibition of glutamate release through inhibition of presynaptic α7 nAChRs presents a powerful mechanism by which KET and its analogs can turn off key glutamatergic outputs throughout the CNS (Fig.7B). This dual inhibitory mechanism probably explains why KET is efficacious in halting nerve agent-induced seizures. There are many examples of NMDAR antagonists that fail to prevent nerve agent-induced seizures. It is also true that selective α7 nAChR antagonists like MLA do not prevent nerve agent-induced seizures.

Inhibition of α7 nAChRs by KET is voltage independent, and, therefore, cannot be explained by open-channel blockade. Also, it does not involve changes in the rapid, agonist-induced desensitization of the receptors, because τ-decays of type IA currents are unaffected.

KET and PCP-induced blockade of α7 nAChRs was very different from the inhibitory effect of kynurenic acid (KYNA), another non-competitive antagonist of both NMDA and α7 nAChRs.[17] First, the onset of KET and PCP inhibition was achieved within 6-8 minutes upon drug application. This was faster than the onset of KYNA inhibition. Second, the slopes of the concentration-response for PCP and KET were what one would expect for an interaction at a single site unlike the shallow slope of the concentration-response for KYNA on α7 nAChRs.[17] In other words, the Hill coefficients for PCP and KET were at unity, in contrast to the negative cooperativity between the binding sites for KYNA on the nAChRs. Thus, PCP and KET are probably not binding to the same site as KYNA. The similarity they do share is their non-competitive voltage-independent mechanism of action. However, β-amyloid$_{1-42}$ and buproprion (zyban) are two examples of non-competitive voltage-independent inhibitors of α7 nAChRs that bind to distinct sites. In the case of β-amyloid$_{1-42}$, the target is believed to be the amino terminal end of the α7 subunits of this nAChR. The KYNA binding site was also determined to be located somewhere on the amino-terminal end of the α7 subunit based on KYNA inhibition of chimeric α7-5HT$_3$ receptors, which possess the extracellularly located amino terminal residues of α7 receptors and the membrane spanning domain of the 5-HT$_3$ ionic channel.[19] In summary, it is clear that α7 nAChRs and NMDARs are two significant targets for the central actions of KET and its synthetic precursor PCP.

5.0 CONCLUSIONS

While KET and PCP are thought to exert their central actions exclusively though inhibition of NMDA-type glutamate receptors, the possibility of action on the neuronal nAChR system has largely been ignored. We demonstrate here physiologically significant inhibition of native neuronal α7 nicotinic receptors expressed in rat hippocampal cultures by KET and the KET analog PCP. Therefore, the present study demonstrates that α7 nAChRs are significant targets for the actions by KET and PCP, the derivative of KET. The physiological relevance of their inhibition on the nicotinic cholinergic and glutamatergic systems is discussed below.

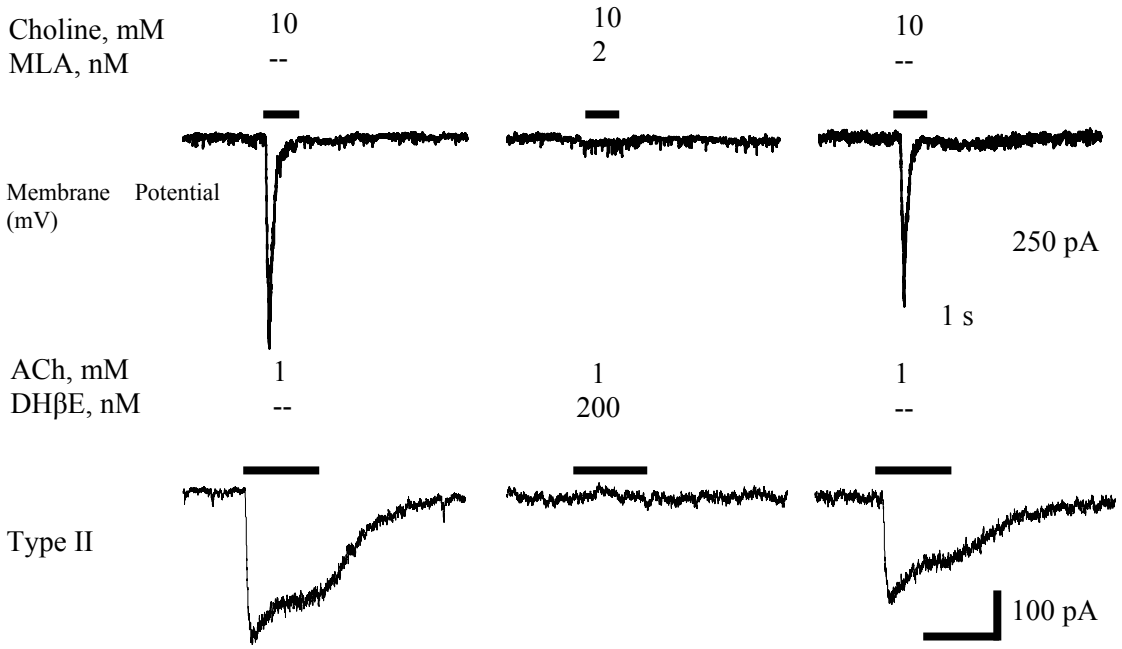

Figure 1. *Native neuronal nAChR currents elicited by choline in cultured hippocampal rat neurons. (Upper Panel).* Methyllycaconitine (MLA) inhibits fast desensitizing ACh-elicited type IA currents, subserved by α7 nAChRs. Currents evoked by U-tube application of ACh are blocked after a 10-min perfusion of the neurons with MLA (2nM)-containing external solution. The effect of MLA is fully reversible after a 15-min washout of the neurons. (*Bottom panel*). Slowly desensitizing currents evoked by U-tube application of ACh (1 mM) are inhibited by DHβE (200 nM)-containing external solution, and the effect of DHβE is fully reversible. All experiments were performed in neurons perfused continuously with external solution containing atropine (1 μM) and TTX (200 nM). Membrane potential, -60 mV.

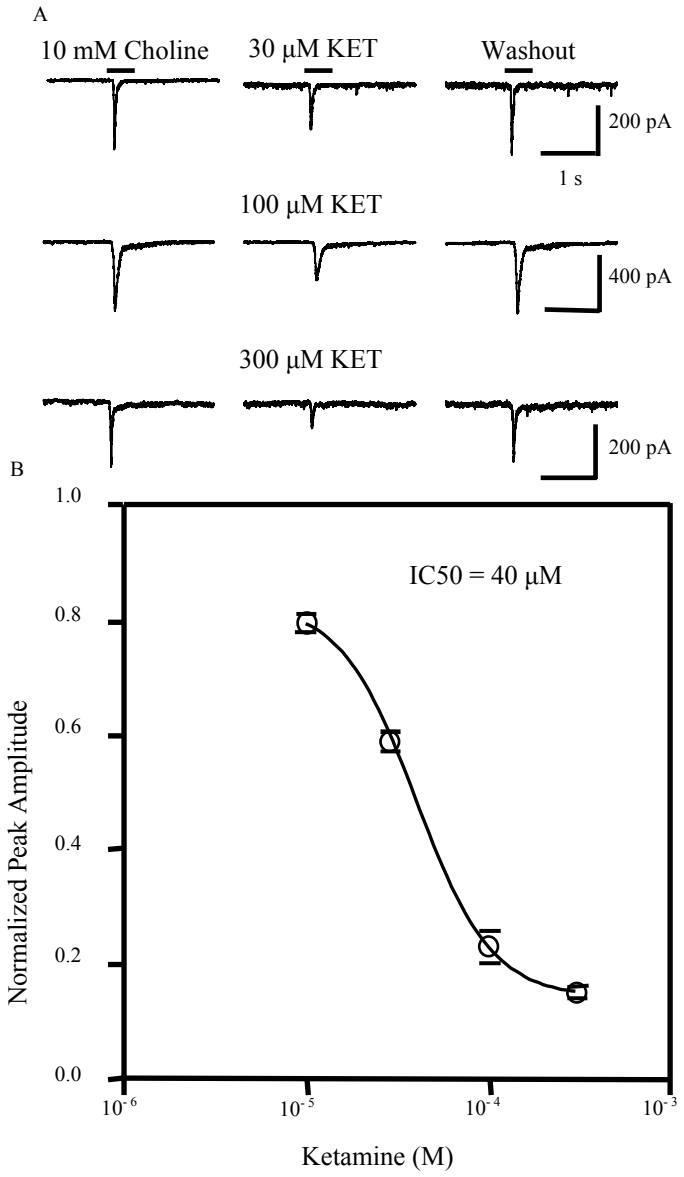

Figure 2. KET-induced blockade of type IA α7 nAChR currents evoked by choline in cultured hippocampal neurons from rat. **A.** *Traces*: Sample recordings of whole-cell currents evoked by choline (10 mM) under control conditions (*left traces*), in the presence of KET (30-300 µM) after perfusion of the neurons with external solution containing KET (*middle traces*), and subsequent washing of the neurons with KET-free external solution (*right traces*). **B.** *Graph*: Semi-logarithmic plot of the concentration-dependent effect of KET on whole-cell currents evoked by choline (10-300 µM). The amplitudes of currents evoked by choline under control condition were taken as unity (1) and used to normalize the amplitudes of currents recorded in the presence of KET. Each point and bar represent mean and S.E.M., respectively, of results obtained from 4-6 experiments. All recordings were obtained in the presence of TTX (200 nM) and atropine (1 µM). Membrane potential, -60 mV.

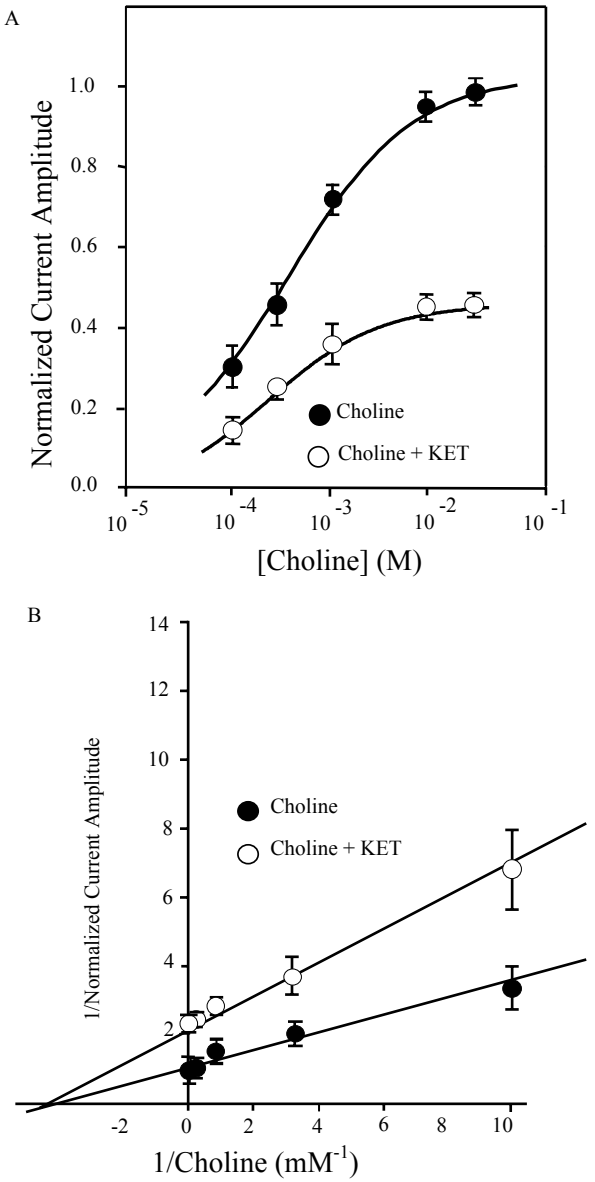

Figure 3. A. Semi-logarithmic plot of the concentration-response relationships for choline in evoking type IA currents in cultured hippocampal neurons in the absence and in the presence of KET. Under the control condition, choline (500 ms pulses; 100 μM – 30 mM) was applied to cultured hippocampal neurons. After recording the control responses evoked by a given concentration of choline, each neuron was perfused for 8 min with external solution containing KET (50 μM) and tested for their responsiveness to pulses of choline plus KET. Finally, a washing of the neuron was performed in the same neuron with KET-free external solution to correct for rundown of current amplitudes. Under the control condition, rundown-corrected amplitudes of choline (30 mM)-evoked currents recorded from hippocampal neurons were taken as 1 and used to normalize the amplitudes of currents evoked by the other concentrations of choline. The EC50 for choline in evoking type IA currents in the absence (●) and in the presence of KET (50 μM; o) was approximately the same, and KET-induced reduction of type IA current amplitudes was insurmountable by increasing choline concentrations. Membrane potential, -60 mV. **B.** Double reciprocal plots of the concentration-response relationships for choline in evoking type IA currents in the absence and in the presence of KET (50 μM). Each symbol and bar represent mean and S.E.M., respectively, of results obtained from 4-6 neurons.

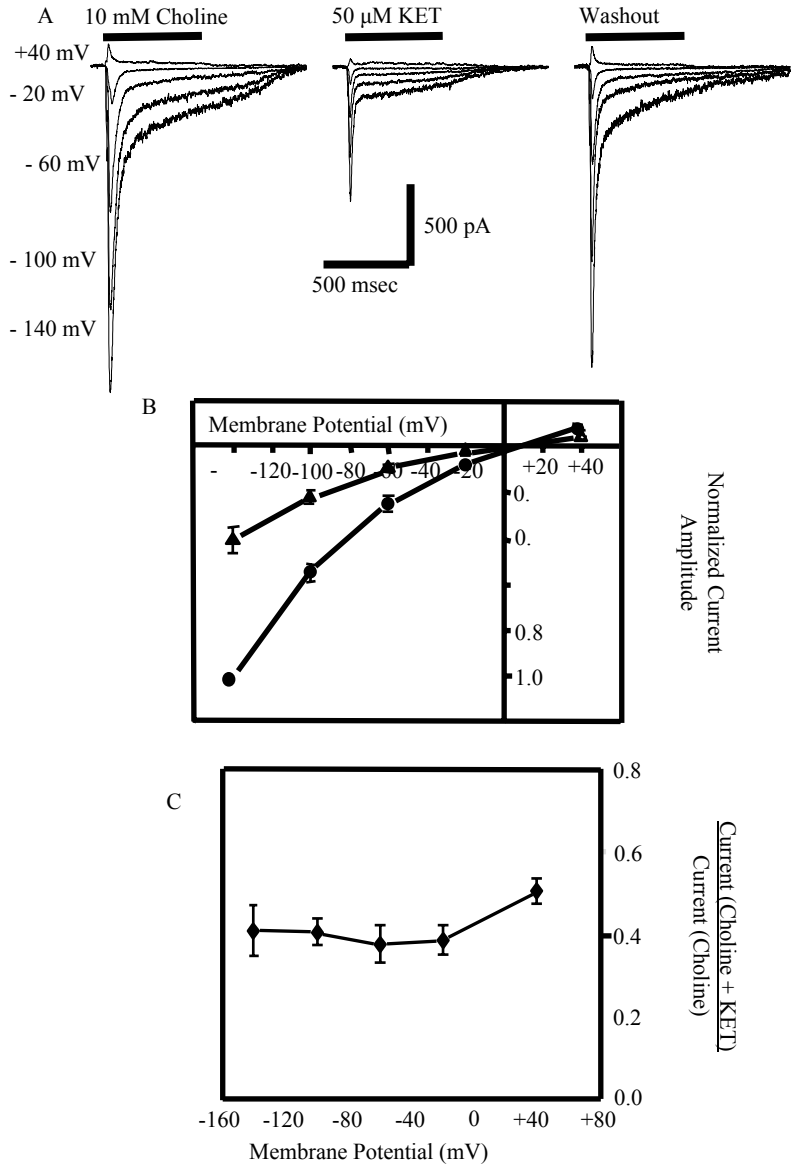

Figure 4. *KET-induced blockade of α7 nAChRs is voltage independent.* **A.** Traces of sample recordings of choline (10 mM)-evoked type IA currents obtained from hippocampal neurons at various holding potentials (-140 mV to +40 mV), before their exposure to KET (50 µM) (left traces), after perfusion with KET-containing external solution (middle traces) and after washing with KET-free external solution (right traces). **B.** Current-voltage relationships for responses evoked by choline (10 mM) in the absence or presence of KET (50 µM). Under the control condition, rundown-corrected amplitudes of choline (10 mM)-evoked currents recorded from neurons voltage clamped at -140 mV were taken as 1 and used to normalize the amplitudes of currents evoked by choline at all other membrane potentials. The plot of the normalized current amplitude versus membrane potential could be fitted by a straight line. Rundown-corrected amplitudes of type IA currents evoked by choline (10 mM) at any membrane potential were taken as 1 and used to normalize the amplitudes of type IA currents evoked by pulses of choline plus KET (50 µM) at that membrane potential. **C.** Plot of the ratio of the amplitudes of currents evoked by pulses of choline plus KET and the amplitudes of currents evoked by choline alone versus membrane potential. Each symbol and bar represent mean and S.E.M., respectively, of results obtained from 6 neurons. All experiments were performed in the presence of TTX (200 nM) and atropine (1 µM).

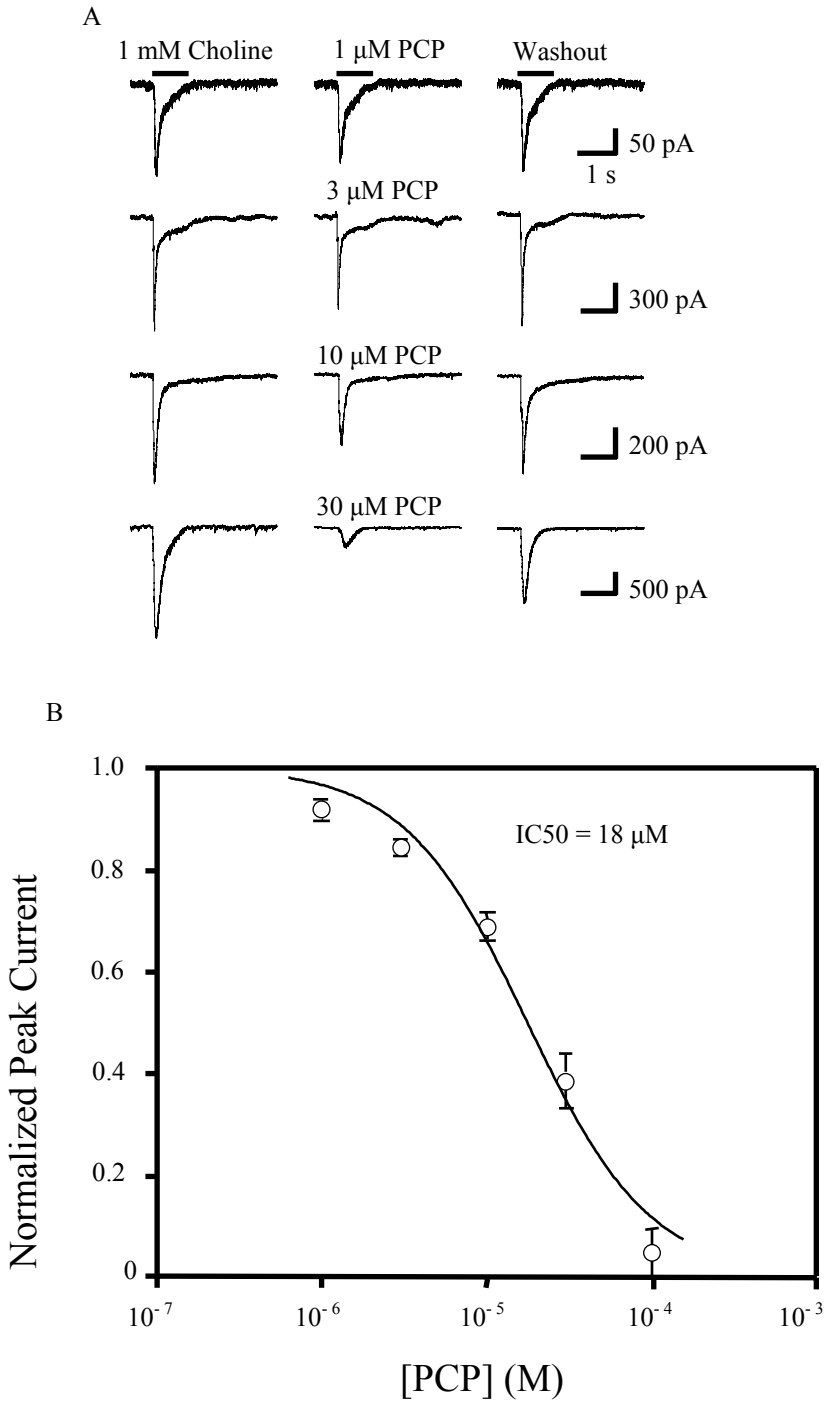

Figure 5. *PCP-induced inhibition of type IA currents in primary cultures of rat hippocampus.* **A.** Sample recordings of choline-evoked type IA currents obtained from hippocampal neurons were exposed to each PCP concentration. **B.** Concentration-response relationship for PCP-induced blockade of choline (1 mM)-evoked type IA currents. The rundown-corrected amplitude of type IA currents was taken as 1 and used to normalize the amplitude of type IA currents evoked by choline in the presence of PCP (1 μM to 100 μM). Each point and bar represent mean and S.E.M., respectively of results obtained from 4 neurons. All experiments were performed in neurons perfused continuously with external solution containing atropine (1 μM) and TTX (200 nM). Membrane potential, -60 mV.

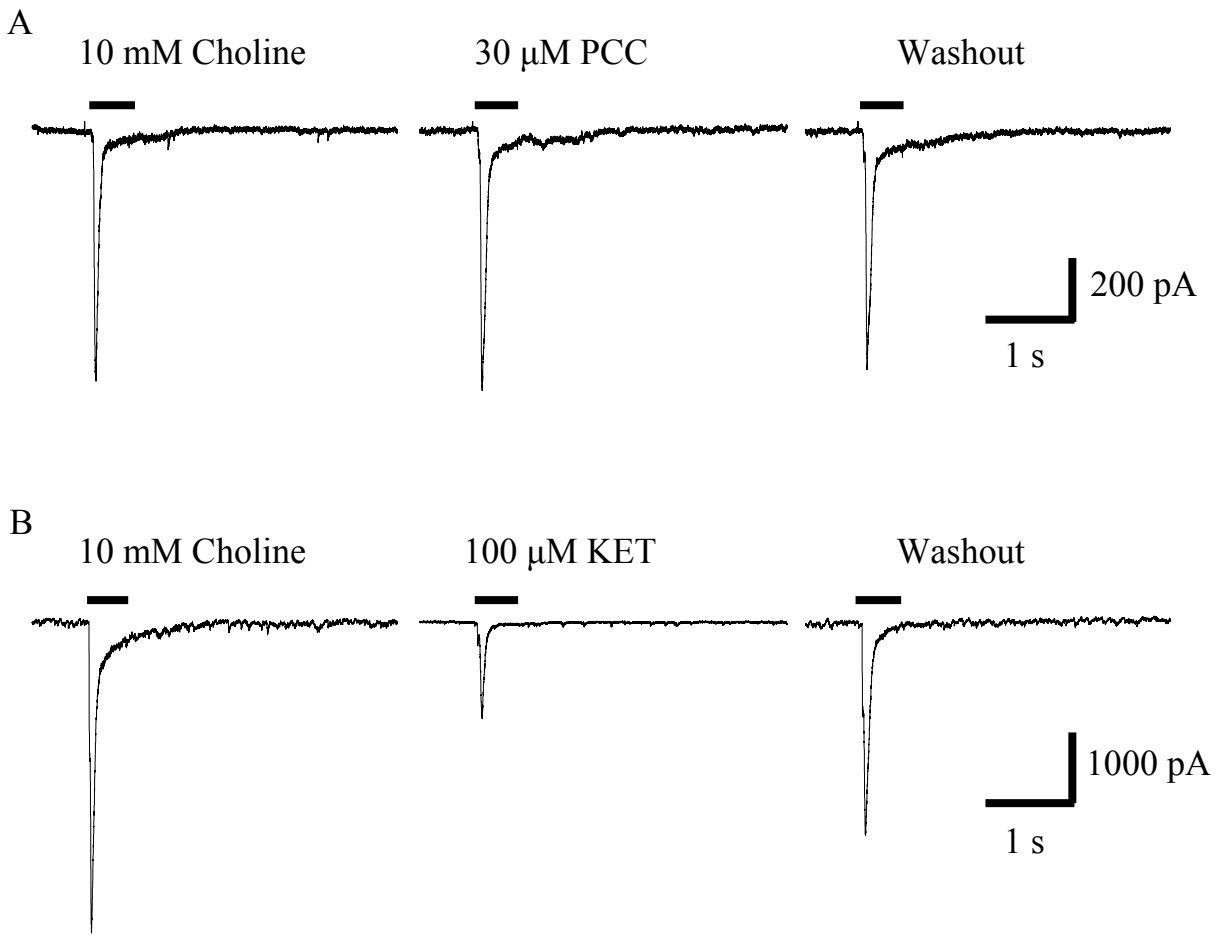

Figure 6. *The behaviorally inactive PCP precursor compound PCC does not inhibit α7 subtype nAChRs*. **A**. PCC does not inhibit type IA currents in cultured hippocampal neurons. Fast desensitizing currents evoked by U-tube application of choline (10 mM) (*left trace*) were unaffected by PCC (30 μM) (*middle trace*). **B**. Sample recordings of choline-evoked type IA currents obtained from hippocampal neurons before their exposure to KET (100 μM; *left trace*), after perfusion with KET-containing external solution (*middle trace*), and after washing with KET-free external solution (*right trace*).

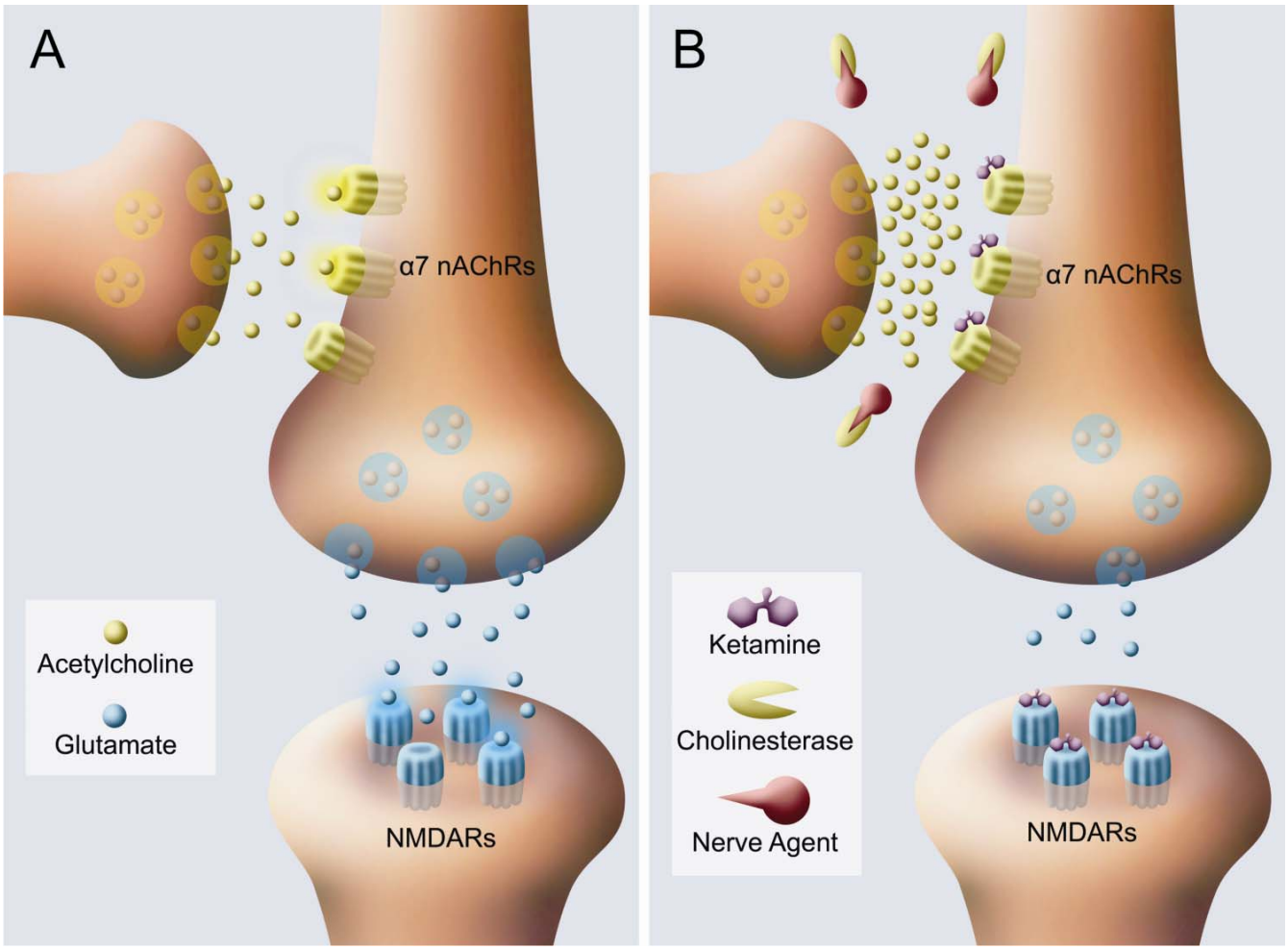

Figure 7. A. (*Left panel*) Illustration depicting the dual mechanism of KET action in the hippocampus. Presynaptic α7 nAChRs function to modulate the release of the excitatory neurotransmitter in the hippocampus. During normal activation, ACh is released from neighboring neurons, activating the α7 subtype nAChRs. Activation of α7 results in release of the excitatory neurotransmitter glutamate, binding to post-synaptic NMDARs and activation. **B.** Nerve agent binds to acetylcholinesterase (AChE), preventing degradation of ACh in the synapse. This results in constant activation of α7 nAChRs and hyperactivation of glutamatergic release. The end result is a major shift to promote excitatory neurotransmission. KET blocks both the nicotinic and glutamatergic components to reduce the excitatory influence caused by nerve agents. KET inhibits α7 nAChRs through a voltage-independent, non-competitive antagonism, reducing the amount of glutamate released. KET also inhibits NMDARs directly to further reduce the glutamatergic input. Both mechanisms serve to halt nerve agent-induced seizures resulting from excitatory neurotransmission. Illustrated by Alexandre M. Katos, USAMRICD.

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