*Current Perspective***Pharmacological Study on Alzheimer's Drugs Targeting Calcium/Calmodulin-Dependent Protein Kinase II**Shigeki Moriguchi^{1,*}¹Department of Pharmacology, Graduate School of Pharmaceutical Sciences, Tohoku University,
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Abstract. In the brain of Alzheimer's disease patients, down-regulation of both cholinergic and glutamatergic systems have been found and is thought to play an important role in impairment of cognition, learning, and memory. Nefiracetam is a pyrrolidine-related nootropic drug exhibiting various pharmacological actions such as a cognitive-enhancing effect. The present study was undertaken to elucidate mechanisms underlying the action of nefiracetam on glutamatergic receptors and intracellular protein kinases. *N*-Methyl-D-aspartate (NMDA)-evoked currents were recorded from rat cortical neurons in long-term cultured primary neurons using the whole-cell patch-clamp technique. NMDA-evoked currents were greatly and reversibly potentiated by bath application of nefiracetam, resulting in a bell-shaped dose–response curve. The maximum potentiation of 170% relative to the control was produced at 10 nM. Treatment with an inhibitor of the glycine binding site of the NMDA receptor, 7-chlorokynurenic acid, at 1 μ M prevented augmentation of NMDA-evoked currents by nefiracetam. In rat hippocampal CA1 slices, field excitatory postsynaptic potentials were recorded by stimulation of Schaffer collateral/commissural pathways. Nefiracetam treatment significantly enhanced long-term potentiation (LTP) with the same bell-shaped dose–response curve. Furthermore, nefiracetam-induced LTP enhancement was closely associated with calcium/calmodulin-dependent protein kinase II (CaMKII) activation with concomitant increase in phosphorylation of AMPA-type glutamate receptor subunit 1 (GluA1) (Ser-831) as a postsynaptic CaMKII substrate. In conclusion, nefiracetam enhances NMDA-receptor function through stimulation of its glycine binding site and nefiracetam-induced CaMKII activation likely contributes to improvement of cognition, learning, and memory.

Keywords: nefiracetam, *N*-methyl-D-aspartate receptor, calcium/calmodulin-dependent protein kinase II, long-term potentiation, cognition

1. Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder marked by progressive loss of memory and cognitive function. It is known that AD is associated with down-regulation of the cholinergic system in the brain (1). Stimulation of the cholinergic system improves the cognitive function. This approach has indeed proven

successful to a limited extent, and the U.S. Food and Drug Administration has approved four anticholinesterase drugs, tacrine, donepezil, rivastigmine, and galantamine for treatment of AD patients. These drugs increase the amount of acetylcholine (ACh) in the synaptic cleft, thereby stimulating the cholinergic system, but they cause some side effects such as nausea, diarrhea, and vomiting. Furthermore, their efficacy in improving cognition, learning, and memory is somewhat limited.

Recently, newer approaches are urgently required. One of these approaches is to directly stimulate neuronal nicotinic acetylcholine receptor (nAChR) in the brain. It

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has indeed been demonstrated that galantamine potentiates ACh-induced currents in nAChR. The optimal concentration of galantamine to maximally potentiate ACh-induced currents is 0.1 to 1 μ M (2, 3). By contrast, we have focused on the novel pyrrolidone derivative nootropic drug nefiracetam as shown in the present study. Nefiracetam also potentiates ACh-induced currents in $\alpha 4\beta 2$ -type nAChR in rat cortical neurons (4). Thus, direct potentiation of nAChR activity is a promising approach.

Reduction of *N*-methyl-D-aspartate receptor (NMDAR) functions is also seen in AD patients, possibly contributing to memory deficits (5). We previously reported that galantamine potentiates NMDAR activity in rat cortical neurons (6). Potentiation of nAChR and enhancement of NMDAR activity may synergistically antagonize deficits in memory and cognitive function in AD. NMDAR-mediated increases in intracellular calcium trigger enhanced synaptic activity known as long-term potentiation (LTP) in the hippocampus (7). As LTP in the hippocampus is thought to be a molecular basis of learning and memory in the mammalian central nervous system (8, 9), potentiation of NMDAR function by galantamine possibly underlies its benefits for cognitive function.

A critical role for activation of calcium/calmodulin-dependent protein kinase II (CaMKII) in LTP induction has been established (10). CaMKII is highly enriched in postsynaptic densities of excitatory synapses and becomes constitutively active through autophosphorylation (10–12). Facilitating synaptic efficacy by CaMKII is associated with up-regulation of postsynaptic α -amino-3-hydroxy-5-methyl-4-isoxazolepropionate type glutamate receptor (AMPA) function by direct phosphorylation (12, 13) and trafficking of AMPAR into postsynaptic membranes (14, 15).

Our working hypothesis is that nootropic drugs improve cognitive function by modulating NMDAR function and CaMKII activity. Therefore, nootropic drugs improve cognitive function by increasing the activity of NMDAR and CaMKII in AD patients.

2. Nefiracetam enhances the glycine binding site of NMDAR function in rat cortical neurons

Figure 1A shows an example of an experiment using rat cortical neurons. NMDA was applied at concentration of 30 μ M to the neuron via a U-tube for 250 ms at an interval of 1 min. Neither Mg^{2+} nor glycine was added to the external solution (16). Bath application of 10 nM nefiracetam greatly potentiated the NMDA-induced current, and the effect was completely reversible after washout with nefiracetam-free solution. The time course of changes in NMDA-induced current amplitude is illustrated in Fig. 1B. Nefiracetam at 10 nM greatly potentiated the NMDA-induced current amplitude to about 170% of the control level (Fig. 1B). Maximum efficacy was obtained at 10 nM nefiracetam in a bell-shaped dose–response relationship (Fig. 1C). 7-Chlorokynurenic acid (7-ClKN), a glycine site blocker, decreased NMDA-induced currents and abolished nefiracetam potentiation of the currents (Fig. 2: A and B). To examine the interaction between nefiracetam and 7-ClKN in a more detailed manner, the dose–response relationship for 7-ClKN to inhibit NMDA-induced currents was determined (Fig. 2C). The IC_{50} values estimated from the dose–response relationship were 295.6 ± 31.1 and 467.6 ± 24.9 nM in the absence and presence of nefiracetam, respectively. Thus, the nefiracetam-induced shift of the dose–response curve in the direction of higher concentrations is consis-

Fig. 1. Nefiracetam potentiates NMDA-induced currents in rat cortical neurons. Currents were evoked at a holding potential of -70 mV by 250-ms applications of 30 μ M NMDA via a U-tube system at an interval of 1 min. A: Currents recorded from rat cortical neurons before, during, and after bath application of 10 nM nefiracetam. B: time course of changes in peak current amplitude before, during, and after bath application of 10 nM nefiracetam. C: a bell-shaped dose–response relationship for nefiracetam potentiation of NMDA currents. * $P < 0.05$, ** $P < 0.01$ vs. the control. Modified from Ref. 16 with permission.

Fig. 2. 7-ClKN, a glycine binding site blocker, prevents nefiracetam potentiation of currents evoked by 30 μ M NMDA. **A:** an example of a series of experiments showing suppression of NMDA current by 1 μ M 7-ClKN. **B:** time course of changes in NMDA current amplitude during bath application of 300 nM and 1 μ M 7-ClKN, 1 μ M 7-ClKN plus 10 nM nefiracetam, and 10 nM nefiracetam alone. **C:** 10 nM nefiracetam shifts the dose-response relationship for 7-ClKN inhibition of currents induced by 30 μ M NMDA in the direction of higher concentration. Modified from Ref. 16 with permission.

tent with the competitive interaction of nefiracetam and 7-ClKN at the glycine site. Drugs that modulate NMDAR-mediated neural transmission by acting at the glycine binding site are potential therapeutic agents to treat memory deficits associated with aging and AD. Both the partial glycine site agonist D-cycloserine and the glycine prodrug milacemide facilitate memory in animal models (17, 18) and have been tested as cognitive enhancers in both healthy subjects and patients with AD.

3. Potentiation of NMDAR-dependent LTP by nefiracetam is associated with CaMKII activation in rat hippocampal CA1 region

We next focused on effect of NMDAR-dependent LTP in the hippocampal CA1 region. In control slices, high frequency stimulation (HFS) of Schaffer collateral/commissural pathways induced LTP, which lasted 60 min (Fig. 3: A and B). Treatment with nefiracetam at 10 or 100 nM in artificial cerebrospinal fluid (ACSF) markedly enhanced LTP, while treatment at 1 or 1000 nM did not (Fig. 3: B and C). Nefiracetam treatment was started 20

min before HFS and continued throughout the experiments. Maximum potentiation of LTP was obtained at 10 nM in a bell-shaped dose-response relationship (Fig. 3: B and C) (19). KN-93, a CaMKII inhibitor at 10 μ M inhibited nefiracetam potentiation of LTP by HFS (Fig. 3B). Consistent with our previous reports (10, 20), increased CaMKII autophosphorylation lasted for at least 60 min after HFS in the hippocampal CA1 region. Similarly, consistent with previous observations (21, 22), phosphorylation of synapsin I (Ser-603) and AMPA-type glutamate receptor subunit 1 (GluA1) (Ser-831) significantly increased following LTP induction and persisted for at least 60 min without changes in the protein levels. Interestingly, LTP induction in the presence of 10 nM nefiracetam further increased CaMKII autophosphorylation and GluA1 (Ser-831) phosphorylation without effect on synapsin I (Ser-603) phosphorylation (Fig. 4). Similar to the effects of nefiracetam on CaMKII activity in the basal condition, nefiracetam treatment markedly potentiated increased CaMKII autophosphorylation in the post-synaptic region, thereby further enhancing the magnitude of LTP.

Fig. 3. Enhancement of LTP by nefiracetam treatment in the hippocampal CA1 region. **A:** typical field excitatory postsynaptic potentials (fEPSPs) recorded from the CA1 region in the presence and absence of 10 nM nefiracetam. **B:** changes in slopes of fEPSPs following HFS in the presence and absence of 10 nM nefiracetam, 10 μ M KN-93. **C:** level of LTP potentiation 60 min after HFS in the presence or absence of nefiracetam. Potentiation by nefiracetam shows a bell-shaped dose-response relationship. ** $P < 0.01$ vs. the control. Modified from Ref. 19 with permission.

Fig. 4. Effects of nefiracetam treatment (10 nM) on increases in CaMKII autophosphorylation and phosphorylation of synapsin I and GluA1 (Ser-831) following LTP induction. **A:** representative images of immunoblots using antibodies against autophosphorylated CaMKII (pCaMKII), CaMKII, phosphorylated synapsin I (Ser-603), synapsin I, phosphorylated GluA1 (Ser-831), and GluA1. Protein levels were unchanged following nefiracetam treatment and LTP induction. **B – D:** quantitative analyses of autophosphorylated CaMKII, synapsin I (Ser-603) and GluA1 (Ser-831) determined by densitometry are summarized. Data are expressed as percentage of the value of controls without HFS in the absence of nefiracetam. ** $P < 0.01$ vs. the control before HFS, †† $P < 0.01$ vs. control without nefiracetam treatment. Modified from Ref. 19 with permission.

4. Conclusion

The discovery of nefiracetam potentiation of NMDAR activity (16) is important for understanding its cognitive enhancing action. The mechanism underlying the bell-shaped dose–response relationship of nefiracetam potentiation of NMDA-induced currents remains to be clarified. Bell-shaped dose–response curves were also obtained in other in vitro studies even involving other receptors (4, 23, 24, and in behavioral experiments with nootropic drugs (25). Therefore, nefiracetam's ability to potentiate the glycine binding site of NMDAR is deemed to contribute to cognitive enhancing action. In fact, D-cycloserine, a partial glycine site agonist, and milacemide, a glycine prodrug, are known to ameliorate memory deficit in animals (17, 18) and have been tested in AD (26–28). Interestingly, nefiracetam enhances NMDAR-dependent LTP by CaMKII stimulation in the hippocampal CA1 region. The effect shows a bell-shaped dose–response relationship in which it peaked at 10 nM. Phosphorylation of GluA1 (Ser-831) by CaMKII underlies increased AMPAR-mediated ionic conductance in LTP (11, 29). Increased phosphorylation of GluA1 (Ser-831) was further potentiated by nefiracetam, suggesting that nefiracetam preferentially activates CaMKII postsynaptically. It is notable that the expression of CaMKII α -containing neurons was selectively lost in the hippocampal CA1 subfield of AD patients (30). It is concluded that nefiracetam potentiation of both the glycine binding site of NMDAR function and CaMKII activity, which are down-regulated in AD patients, account for its cognitive enhancing action.

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