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Potentiation of N-Methyl-D-Aspartate-Induced Currents by the Nootropic Drug Nefiracetam in Rat Cortical Neurons

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Running title: Nefiracetam on NMDA receptors

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ABBREVIATIONS: NMDA, N-methyl-D-aspartate; ACh, acetylcholine; nACh, nicotinic acetylcholine; AMPA, α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid; 7-ClKN, 7-chlorokynurenic acid

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ABSTRACT

Nefiracetam is a new pyrrolidone nootropic drug being developed for the treatment of Alzheimer's type and post-stroke vascular-type dementia. In the brain of Alzheimer's disease patients down-regulation of both cholinergic and glutamatergic systems has been found and is thought to play an important role in impairment of cognition, learning and memory. We have previously shown that the activity of neuronal nicotinic actylcholine receptors is potently augmented by nefiracetam. The present study was undertaken to elucidate the mechanism of action of nefiracetam on glutamatergic receptors. Currents were recorded from rat cortical neurons in long-term primary culture using the whole-cell patch clamp technique at a holding potential of –70 mV in Mg^{2+}-free solutions. NMDA-evoked currents were greatly and reversibly potentiated by bath application of nefiracetam resulting in a bell-shaped dose-response curve. The minimum effective nefiracetam concentration was 1 nM, and the maximum potentiation to 170% of the control was produced at 10 nM. Nefiracetam potentiation occurred at high NMDA concentrations that evoked the saturated response, and in a manner independent of NMDA concentrations ranging from 3-1000 μM. Glycine at 3 μM potentiated NMDA currents but this effect was attenuated with an increasing concentration of nefiracetam from 1 nM to 10,000 nM. 7-Chlorokynurenic acid at 1 μM prevented nefiracetam from potentiating NMDA currents. Nefiracetam at 10 nM shifted the dose-response relationship for the 7-chlorokynurenic acid inhibition of NMDA currents in the direction of higher concentrations. AMPA- and kainate-induced currents were not significantly affected by application of 10 nM nefiracetam. It was concluded that nefiracetam potentiated NMDA currents through interactions with the glycine binding site of the NMDA receptor.

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It is well established that accumulation of β-amyloid in the brain is a hallmark of Alzheimer's disease. However, no strategies for curing the disease have been developed yet as we do not know the exact cause of the disease (Hirai, 2000). It is also known that Alzheimer's disease is associated with down-regulation of the cholinergic system in the brain (Giacobini, 2000). Thus, stimulation of the cholinergic system may improve the patient's cognition, learning and memory. This approach has indeed proven successful to a limited extent, and FDA has approved four anticholinesterases, tacrine, donepezil, revastigmine, and galantamine, for the treatment of Alzheimer's disease patients. These drugs, being anticholinesterases, increase the amount of acetylcholine (ACh) in the synaptic cleft thereby stimulating the cholinergic system, yet they cause some side effects such as nausea, diarrhea, and vomiting. Furthermore, their efficacy in improving cognition, learning and memory is somewhat limited.

Under the circumstances, newer approaches are urgently required. One of these approaches is to directly stimulate neuronal nicotinic acetylcholine (nACh) receptors in the brain. It has indeed been demonstrated recently that nefiracetam (Zhao et al., 2001) and galantamine (Schrattenholz et al., 1996; Maelike and Albuquerque 2000; Maelicke et al., 2001) potentiate ACh-induced currents in nACh receptors. Although galantamine does inhibit cholinesterase, its potency is low. The optimal concentration of galantamine to maximally potentiate ACh-induced currents is 0.1 - 1 μM (Schrattenholz et al., 1996; Maelike and Albuquerque 2000; Maelicke et al., 2001; Santos et al., 2002). Nefiracetam is extremely potent (0.1 –1.0 nM), and it efficaciously potentiates ACh-induced currents to 200% of the control in the α4β2-type ACh receptors in rat cortical neurons (Zhao et al., 2001). Thus, direct potentiation of nACh receptor activity is a promising approach.

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Alzheimer's disease is a progressive neurodegenerative disorder of cognitive function. Reductions in N-methyl-D-aspartate (NMDA) receptors are also found in the brain of Alzheimer's patients, possibly contributing to memory deficits (Greenamyre et al., 1987; Fonnum et al., 1995; Sze et al., 2001; Cowburn et al., 1990). Drugs that modulate NMDA receptor-mediated neural transmission by acting at the glycine binding site of NMDA receptors are potential therapeutic agents to treat memory deficits associated with aging and Alzheimer's disease. Both the partial glycine site agonist d-cycloserine and the glycine prodrug milacemide prevent memory deficit in animal paradigms (Baxter et al., 1994; Hanndelmann et al., 1989), and have been tested as cognitive enhancers in both healthy subjects and patients with Alzheimer's disease (Schwartz et al., 1991, 1996; Dysken et al., 1992). Thus, NMDA receptors play a crucial role in learning and memory. Our working hypothesis is that one of the mechanisms by which nootropic drugs improve cognitive function is by increasing the activity of NMDA receptors as well as nACh receptors in the brain of Alzheimer's disease patients who have down-regulated NMDA receptors. In post-stroke patients who have excess glutamate release, nootropic drugs, being partial agonists, reduce the excess activation of NMDA receptors.

We have found in the present study that nefiracetam potently augments NMDA-evoked currents in rat cortical neurons. This action appears to be exerted via nefiracetam interactions with the glycine binding site on the NMDA receptors.

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Materials and Methods

Cell Preparation. Rat cortical neurons were cultured by a procedure slightly modified from that described previously (Marszalec and Narahashi, 1993). In brief, rat embryos were removed from 17-day pregnant Sprague-Dawley rats under halothane anesthesia. Small wedges of frontal cortex were excised and subsequently incubated in the phosphate buffer solution for 20 min at 37°C. This solution contained 154 mM NaCl, 1.05 mM KH_2 $P0_4$, 3.0 mM $Na_2HP0_4 \cdot 7H_20$, 0.25% (w/v) trypsin (Type XI; Sigma, St. Louis, MO), pH 7.4, and with osmolarity of 287 m0sM. The digested tissue was then mechanically triturated by repeated passages through a Pasteur pipette, and the dissociated cells were suspended in neurobasal medium with B-27 supplement (Life Technologies, Gaithersburg, MD) and 2 mM glutamine. The cells were added to 35-mm culture wells at a concentration of 100,000 cells/ml. Each well contained five 12-mm poly-L-lysine-coated coverslips overlaid with confluent glia that had been plated 2 to 4 weeks earlier. The cortical neurons/glia cultures were maintained in a humidified atmosphere of 90% air and 10% CO_2 at 34°C. Cells cultured for 3 to 5 weeks were used for experiments.

Solutions for Current Recording. The external solution for recording whole-cell currents contained 150 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, 5.5 mM HEPES acid, 4.5 mM HEPES sodium, and 10 mM D-glucose. Tetrodotoxin (100 nM) and atropine sulfate (20 nM) were added to eliminate the voltage-gated sodium channel and the muscarinic ACh receptor currents, respectively. No glycine was added to the external solution unless otherwise stated. The pH was 7.3 and the osmolarity was adjusted to 300 mOsM with D-glucose. The internal pipette solution contained 140 mM potassium-gluconate, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM HEPES acid, 10 mM EGTA, 2 mM ATP-

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Mg^{2+}, and 0.2 mM GTP-Na^{+}. The pH was adjusted to 7.3 with KOH and the osmolarity was adjusted to 300 mOsM by adding D-glucose.

Whole-Cell Current Recording. Ionic currents were recorded using the whole-cell patch clamp technique at room temperature (21-22°C). Pipette electrodes were made from 1.5 mm (outer diameter) borosilicate glass tubes with a resistance of 2 to 3 MΩ when filled with the internal solution. The membrane potential was clamped at -70 mV. The whole-cell current recording started 5 to 10 min after membrane rupture in order that the cell interior adequately equilibrates with the pipette solution. Currents through the electrode were recorded with an Axopatch-1C amplifier (Axon Instruments, Union City, CA), filtered at 2 kHz, and sampled at 10 kHz in a PC-based data acquisition system that also provided preliminary data analysis. Results are expressed as mean ± S.D., and n represents the number of the cells examined.

Drug Application. Two methods for drug application were used: One was application via a U-tube system controlled by a computer-operated magnetic valve system (Marszalec and Narahashi, 1993). The external solution surrounding the cell could be completely changed with the drug solution within 30 to 40 ms. The other method was perfusion through the bath. Test drugs were added to the external solution and continuously perfused to the recording chamber via a glass syringe and Teflon tubes.

Chemicals. NMDA(Sigma), α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) (RBI, Natick, MA), kainate (RBI) and glycine (RBI) were first dissolved in distilled water to make stock solutions. Tetrodotoxin and atropine sulfate were purchased from Sigma. Nefiracetam [DM-9384; N-(2,6-dimethylphenyl)-2-(2-oxo-l-pyrrolidinyl) acetamide] was provided by Daiichi Pharmaceutical Company (Tokyo, Japan), and was

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first dissolved in distilled water to make stock solutions. The stock solutions were stored at 4°C and diluted to prepare test solutions with the standard external solution shortly before the experiments.

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Results

Potentiation of NMDA-induced currents by nefiracetam. Rat cortical neurons in long-term primary culture comprised at least three types of cells: pyramidal neurons, multipolar neurons, and bipolar nuerons. Whereas the three types of neurons generated currents in response to NMDA application, the effects of nefiracetam on NMDA-induced currents were different. NMDA currents of pyramidal and multipolar neurons were potentiated by nefiracetam, whereas those of bipolar neurons were not affected at all.

Figure 1A shows an example of an experiment using a multipolar neuron. NMDA was applied at a concentration of 30 μM 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. Bath application of 10 nM nefiracetam greatly potentiated the NMDA-induced current, and the effect was completely reversible after washing with nefiracetam-free solution. The time course of changes in NMDA current amplitude during and after bath application of 10 nM nefiracetam is illustrated in Fig. 1B. The current amplitude was increased by nefiracetam to ~1700 pA from the control level of ~1100 pA, representing about 50% increase. In contrast, smaller bipolar neurons did not respond to 10 nM nefiracetam, the NMDA current amplitude remaining nearly constant during and after bath perfusion of nefiracetam (Fig. 1C and D). All of the subsequent experiments were performed using multipolar neurons unless otherwise stated.

Dose-response relationships for nefiracetam potentiation of NMDA-induced currents are shown in Fig. 2. The minimal effective concentration of nefiracetam to potentiate NMDA currents in multipolar cells was 1 nM at which only about 10% current potentiation over and above control was observed (Fig. 2A). Nefiracetam at 10 nM greatly

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potentiated the current by 70%, yet further increases in the concentration to 100 nM and 1000 nM caused a decrease in efficacy (Fig. 2A). This resulted in a bell-shaped dose-response relationship with a maximal efficacy at 10 nM nefiracetam (Fig. 2B, □).

Whereas the mechanism that underlies the lack of NMDA response of bipolar neurons to the potentiating action of 10 nM nefiracetam remains to be seen, there is a possibility that bipolar neurons have a much lower sensitivity to nefiracetam than multipolar neurons. However, this possibility was ruled out by the experiments using bipolar neurons which showed no potentiation by nefiracetam even at higher concentrations up to 1000 nM (Fig. 2B, □).

Nefiracetam potentiation is independent of NMDA concentration. At least two possible mechanisms of nefiracetam potentiation of NMDA currents are conceivable. One is an increase in the affinity of the receptor for NMDA causing a shift of NMDA dose-response curve in the direction of lower concentrations of NMDA. The other possibility to potentiate NMDA currents is an increase in current amplitude even at high concentrations of NMDA that produce a saturating response, without causing a shift of NMDA dose-response curve. The dose-response relationships for NMDA-induced currents before and during bath perfusion of 10 nM nefiracetam are illustrated in Fig. 3A. The peak current amplitudes normalized to the maximum control current induced by 1000 μM NMDA were fitted by a sigmoid curve with an EC_{50} of 36.9 ± 0.3 μM and a Hill coefficient of 0.58 ± 0.07 (Fig. 3A, ○, n=4). After a 10 min bath perfusion of 10 nM nefiracetam, the maximal current amplitude induced by 1000 μM NMDA was 148.2 ± 7.5% of the control maximum, an EC_{50} was 28.2 ± 0.2 μM, and a Hill coefficient was 0.66 ± 0.08 (Fig. 3A, ●, n=4). Thus, nefiracetam caused a slight shift of NMDA dose-response curve in the

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direction of lower concentrations, and a large increase in maximal current amplitude resulting in an increase in the saturating response evoked by 1000 μM NMDA. Therefore, the latter mechanism is mainly responsible for nefiracetam potentiation of NMDA currents.

The current amplitudes relative to the control during perfusion with nefiracetam are plotted as a function of NMDA concentrations in Fig. 3B. Nefiracetam at 10 nM significantly potentiated currents at all NMDA concentrations in a manner independent of NMDA concentrations.

Nefiracetam-glycine interactions at the NMDA receptor. One of the possible sites of action of nefiracetam in the NMDA receptor is the glycine binding site. To examine this possibility, interactions of glycine and nefiracetam at the NMDA receptor were studied in the absence of Mg^{2+} ions in the external solution. The NMDA dose-response relationship in the presence of 3 μM glycine in the external solution is shown in Fig. 3A (▲). The current was greatly potentiated by glycine, and the data were fitted by a sigmoid curve with the maximal current of 159.8 ± 11.7% of the control maximum, an EC_{50} of 26.7 ± 0.3 μM, and a Hill coefficient of 0.75 ± 0.05 (Fig. 3A, ▲, n=4). Addition of 10 nM nefiracetam to the solution containing 3 μM glycine resulted in a decrease in current amplitude from the level of 3 μM glycine alone. The current amplitudes were fitted by a sigmid curve with the maximal current of 136.5 ± 9.2% of the control maximum, an EC_{50} of 30.0 ± 0.2 μM, and a Hill coefficient of 0.70 ± 0.07 (Fig. 3A, △, n=4). Therefore, the ability of 3 μM glycine in potentiating NMDA currents was attenuated by 10 nM nefiracetam.

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The interactions between glycine and nefiracetam at the NMDA receptor are more clearly shown in the experiments in which neifracetam was tested at various concentrations (Fig. 4). After confirming the potentiating action of 3 μM glycine on NMDA currents, nefiracetam was applied to the bath at various concentrations ranging from 1 nM to 10,000 nM. The glycine-induced potentiation was reduced with increasing concentration of nefiracetam, almost disappeared at 100 nM nefiracetam, and was reversed at 1,000 and 10,000 nM nefiracetam showing slight suppression beyond the level of control. These effects were reversible after washing with solution free of glycine and nefiracetam. These results lead to the suggestion that nefiracetam binds to the glycine binding site of the NMDA receptor.

The interactions between glycine and nefiracetam were further studied using various concentrations of glycine. A series of experiments illustrated in Fig. 5A indicated that although glycine, when applied via the U-tube, potentiated NMDA currents in a concentration-dependent manner until the current amplitude reached a maximum at 3000 nM glycine, the same protocol, but in the presence of 10 nM nefiracetam in the bath, caused attenuation of current amplitude over and above that at 100 nM glycine. Thus, nefiracetam at 10 nM abolished potentiation caused by high concentrations of glycine at 1000 and 3000 nM. These results suggest that nefiracetam binds to the glycine site with higher affinity but with lesser efficacy than glycine.

The data shown in Fig. 5A appeared to be inconsistent with the partial agonist hypothesis, because glycine at high concentrations did not overcome the effect of nefiracetam. However, in this experiment, glycine was co-applied via the U-tube with glutamate. There might not be sufficient time for glycine to compete with bath applied

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nefiracetam for the glycine site, consistent with our previous conclusion that nefiracetam is a more potent but less efficacious agonist than glycine.

To further pursue this problem, the experiment shown in new Fig. 5B was carried out under equilibrium condition where glycine was applied both through the bath and via the U-tube. Thus, glycine would have ample time to compete with nefiracetam for the glycine site. Glycine at high concentrations was indeed able to compete with nefiracetam and the maximum effect of glycine was not reduced by the presence of nefiracetam.

7-Chlorokynurenic acid abolishes nefiracetam potentiation of NMDA currents. The aforementioned results suggest that nefiracetam acts on the glycine binding site of the NMDA receptor thereby potentiating NMDA-induced currents. In order to prove the validity of this hypothesis, 7-chlorokynurenic acid (7 ClKN) which is known to be a glycine binding site antagonist was used. 7 ClKN applied to the bath at a concentration of 300 nM did not change the amplitude of current evoked by 30 μM NMDA (Fig. 6a and b). However, 7-ClKN at 1 μM greatly suppressed the NMDA current (Fig. 6c). In the presence of 1 μM 7-ClKN in the bath, nefiracetam at 10 nM no longer caused potentiation of NMDA current (Fig. 6d). Washing with drug-free solution restored the current (Fig. 6e), and bath perfusion of 10 nM nefiracetam again potentiated the current (Fig. 6f) reversibly (Fig. 6g). Thus, it was concluded that nefiracetam binds to the glycine site of the NMDA receptor thereby causing a potentiation of NMDA-induced currents.

To examine the interaction between nefiracetam and 7-ClKN in a more detailed manner, the dose-response relationship for 7-ClKN to inhibit NMDA currents was determined before and during application of 10 nM nefiracetam (Fig. 7). The IC_{50} values estimated from the dose-response relationships were 295.6 ± 31.1 and 467.6 ± 24.9 nM in

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the absence and presence of nefiracetam, respectively. Thus, the nefiracetam-induced shift of the dose-response curve in the direction of higher concentrations is consistent with the competitive interaction of nefiracetam and 7-ClKN at the glycine site.

Effects of nefiracetam on AMPA and kainate currents. Besides the NMDA current, rat cortical neurons in primary culture generated currents in response to U-tube application of AMPA or kainate. AMPA- and kainate-induced currents were not significantly affected by nefiracetam. Figure 8A and B shows that 10 nM nefiracetam slightly potentiated currents induced by 30 μM AMPA, but the effect was not significant. Fig. 8C and D shows that currents induced by 30 μM kainate were not affected at all by 10 nM nefiracetam. It was concluded that among the three subtypes of glutamate receptors, the AMPA and kainate receptors are not responsive to any modulating action of nefiracetam.

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Discussion

We have previously reported that nefiracetam augments the α4β2-type nACh receptor currents in a highly potent (~1 nM) and efficacious (to ~200% of control) manner (Zhao et al., 2001). The present study demonstrates the NMDA receptor to be another target highly sensitive to the potentiating action of nefiracetam. NMDA-induced currents in rat cortical neurons in primary culture were potentiated by nefiracetam following a bell-shaped dose-response curve with the maximal potentiation occurring at 10 nM. Evidence was obtained to indicate that nefiracetam acted on the glycine binding site of the NMDA receptor. Nefiracetam had little or no effect on AMPA- or kainate-induced currents.

It is well known that in the brain of Alzheimer's patients the NMDA receptor as well as the nACh receptor is down-regulated. The role of nACh receptors in Alzheimer's patients received much attention (Giacobini, 2000), and four anticholinesterases have been developed into clinical use to improve cognition/learning/memory of the patients through stimulation of the cholinergic system. However, these anticholinesterases cause side effects characteristics of cholinesterase inhibition and the therapeutic effects are not long-lasting. Thus, other ways of improving the patient's conditions need to be sought. Direct stimulation of nACh receptors without inhibiting cholinesterase seems to be a logical approach, and nefiracetam exerts exactly that action. It seems also helpful if a drug stimulates both nACh receptors and NMDA receptors. We have now found that nefiracetam potentiates NMDA currents as well as ACh currents. Although overstimulation of NMDA receptors would cause cell death via excess influx of Ca^{2+} ions, down-regulation of NMDA receptors would have deteriorative effects especially on learning and memory (Brown et al., 1997). Thus, potentiation of NMDA receptor activity,

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which is suppressed in the brain of Alzheimer's patients, up to the normal level is deemed therapeutic.

Nefiracetam was found to interact with the glycine binding site of the NMDA receptor. As expected from its higher affinity but lesser efficacy than glycine, nefiracetam potentiates NMDA currents in the nominal absence of glycine or in the presence of low concentrations of glycine, it suppresses the NMDA currents augmented by higher concentrations of glycine. This will prevent the NMDA receptor from being overactivated. In fact, d-cycloserine, a partial glycine site agonist, is known to improve implicit memory performace of words in Alzheimer's patients (Schwartz et al., 1996) and reversal learning of rat in the water maze (Rickkinen et al., 1998). Thus, nefiracetam and d-cycloserine, as the glycine site partial agonists, share the common mechanism of action with respect to stimulation of NMDA receptors.

The mechanism underlying a bell-shaped dose-response curve for nefiracetam potentiation of NMDA currents is unknown. However, a similar bell-shaped dose-response curve was found in nefiracetam potentiation of ACh currents (Zhao et al., 2001); in nefiracetam potentiation of high voltage-gated calcium channel currents (Yoshii and Watabe, 1994); in GABA release (Watabe et al., 1993); and even in improvement of learning and memory in rat behavioral experiments (Sakurai et al., 1989). The mechanism of bell-shape dose-response curves awaits further experimentation.

Morphological features of the cells dissociated from 15-day gestational age rat fetal cortex after 4 to 6 weeks of culture were found similar to those of cortical neurons in situ: neurons were characterized by their cell forms such as pyramidal, multipolar, fusiform, etc. (Kriegstein and Dichter, 1983). Responses to nefiracetam as revealed in the present study

were different depending on the cell type. NMDA currents recorded from pyramidal and multipolar neurons were potentiated by nefiracetam, whereas the currents from bipolar neurons were not affected by nefiracetam. One possible reason for this differential action is the differences in NMDA receptor subtypes. The NMDA receptor comprises one or more of the four NR2 subunits, NR2A, NR2B, NR2C, and NR2D, together with at least one NR1 subunit (Sucher et al., 1996). The NR1 and NR2B subunits display high levels of expression during the first week of development. In contrast, the expression of the NR2A subunit increases gradually reaching the matured levels by 21 days in culture (Li et al., 1998). Thus, the subunit composition of the NMDA receptor including the NR2A and NR2B subunits may be different between pyramidal/multipolar type neurons and bipolar type neurons exhibiting differential sensitivity to nefiracetam.

Another possibility is that the second messenger system, specifically that related to phosphorylation and dephosphorylation, might be different among these neurons. The NMDA receptor is known to be modulated by phosphorylation. Further experiments are needed to explore this possibility.

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References

Baxter MG, Lanthorn TH, Frink KM, Golski S, Wan R and Olton DS (1994) d-Cycloserine, a novel cognitive enhancer, improves spatial memory in aged rats. *Neurobiol Aging* **15**:207-213.

Brown, DR, Wyper DJ, Owens J, Patterson J, Kelly RC, Hunter R and McCulloch J (1997) ^{123} Iodo-MK801: a spect agent for imaging the pattern and extent of glutamate (NMDA) receptor activation in Alzheimer's disease. *J. Psychiat. Res*. 31:605-619.

Cowburn, RF, Hardy JA, and Roberts PJ (1990). Glutamatergic neurotransmission in Alzheimer's disease. *Biochem. Soc. Trans.* 18:390-392.

Dysken, MW, Mendels J, and LeWitt P (1992) Milacemide; a placebo-controlled study in senile dementia of the Alzheimer type. *J Am Geriatr Soc* **40**:503-506.

Fonnum, F, Myhrer T, Paulsen RE, Wangen K, and Oksengard AR. (1995). Role of glutamate and glutamate receptors in memory function and Alzheimer's Disease. Ann. N.Y. Acad. Sci. 757:475-486.

Giacobini E (2000) Cholinesterase inhibitor therapy stabilizes symptoms of Alzheimer Disease. Alzheimer Dis Assoc Disorders **14** (Suppl 1):S3-S10.

Greenamyre JT, Penney JB, D'Amato CJ and Young AB (1987) Dementia of the Alzheimer's type: changes in hippocampal L-[^{3}H]glutamate binding. *J Neurochem* **48**:543-551.

Hanndelmann GE, Nevins ME, Mueller LL, Arnolde SM and Cordi AA (1989) Milacemide, a glycine prodrug, enhances performance of learning tasks in normal and amnestic rodents. *Pharmacol Biochem Behav* **34**:823-828.

Hirai S (2000) Alzheimer Disease: Current therapy and future therapeutic strategies. *Alzheimer Dis Assoc Disorders* **14** (Suppl. 1):S11-S17.

Kriegstein AR and Dichter MA (1983) Morphological classification of rat cortical neurons in cell culture. *J Neurosci* **3**:1634-1647.

Li JH, Wang VH, Wolfe BB, Kruegger KE, Corsi L, Stocca G and Vicini S (1998) Developmental changes in localization of NMDA receptor subunits in primary cultures of cortical neurons. *Eur J Neurosci* **10**:1704-1715.

Maelicke A and Albuquerque EX (2000) Allosteric modulation of nicotinic acetylcholine receptors as a treatment strategy for Alzheimer's disease. *Eur J Pharmacol* **393**:165-170.

Maelicke A, Samochocki M, Jostock R, Fehrenbacher A, Ludwig J, Albuquerque EX and Zerlin M (2001) Allosteric sensitization of nicotinic receptors by galantamine, a new treatment strategy for Alzheimers's disease. *Soc Biol Psychiatry* **49**:279-288.

Marszalec W and Narahashi T (1993) Use-dependent pentobarbital block of kainate and quiaqualate currents. *Brain Res* **608**:7-15.

Riekkinen P Jr, Ikonen S and Riekkinen M (1998) D-cyloserine, a partial NMDA receptor-associated glycine-B site agonist, enhances reversal learning, but a cholinesterase inhibitor and nicotine has no effect. *NeuroReport* **9**:3647-3651.

Sakurai T, Ojima H, Yamasaki T, Kojima H and Akashi A (1989) Effects of n-(2,6-dimethylphenyl)-2-(2-oxo-1-pyrrolidinyl) acetamide (DM-9384) on learning and memory in rats. *Japan J Pharmacol* **50**:47-53.

Santos MD, Alkondon M, Pereira EFR, Aracava Y, Eisenberg HM, Maelicke A and Albuquerque EX (2002) The nicotinic allosteric potentiating ligand galantamine facilitates synaptic transmission in the mammalian central nervous system. *Mol Pharmacol* **61**:1222-1234.

Schrattenholz A, Pereira EFR, Roth U, Weber K-H, Albuquerque EX and Maelicke A (1996) Agonist responses of neuronal nicotinic acetylcholine receptors are potentiated by a novel class of allosterically acting ligands. *Mol Pharmacol* **49**:1-6.

Schwartz BL, Hashtroudi S, Herting RL, Handerson H and Deutsh SI (1991) Glycine prodrug facilitates memory retrieval in humans. *Neurology* **41**:1341-1343.

Schwartz BL, Hashtroudi S, Herting RL, Schwartz BA and Deutsh SI (1996). d-Cycloserine enhances implicit memory in Alzheimer patients. *Neurology* **46**:420-424.

Sucher NJ, Awobuluyi M, Choi YB and Lipton SA (1996) NMDA receptors: From genes to channels. *Trends Pharmacol Sci* **17**:348-355.

Sze, C-I, Bi H, Kleinschmidt-DeMasters BK, Filley CM, and Martin LJ (2001). N-Methyl-D-aspartate receptor subunit proteins and their phosphorylation status are altered selectively in Alzheimer's disease. *J. Neurolog. Sci*. 182:151-159.

Watabe S, Yamaguchi H and Ashida S (1993) DM-9384, a new cognition-enhancing agent, increases the turnover of components of the GABAergic system in the rat cerebral cortex. *Eur J Pharmacol* **238**:303-309.

Yoshii M and Watabe S (1994) Enhancement of neuronal calcium channel currents by the nootropic agent, nefiracetam (DM-9384), in NG108-15 cells. *Brain Res* **642**:123-131.

Zhao X, Kuryatov A, Lindstrom JM, Yeh JZ and Narahashi T (2001) Nootropic drug modulation of neuronal nicotinic acethylcholine receptors in rat cortical neurons. *Mol Pharmacol* **59**:674-683.

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Footnotes

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Figure Legends

Fig. 1. Nefiracetam potentiates NMDA-induced currents in multipolar type neurons (30-60 μm in diameter) but not in bipolar type neurons (15-30 μm in diameter). Currents were evoked at a holding potential of –70 mV by 250-ms applications of 30 μM NMDA via a U-tube system at an interval of 1 min. A. Currents recorded from a multipolar neuron before (a), during (b), and after (c) bath application of 10 nM nefiracetam. B. Time course of changes (mean ± S.D., n=5) in peak current amplitude before, during, and after bath application of 10 nM nefiracetam in multipolar neurons. C. Currents recorded from a bipolar neuron before (a), during (b), and after (c) bath application of 10 nM nefiracetam. Nefiracetam had no effect on the current. D. Time course of changes (mean ± S.D., n=5) in peak current amplitude before, during, and after bath application of 10 nM nefiracetam in bipolar neurons. Nefiracetam had no effect on the current.

Fig. 2A. Dose-response relationships of nefiracetam potentiation of NMDA-induced currents in multipolar neurons. Currents were evoked by 30 μM NMDA at a holding potential of –70 mV, and nefiracetam was applied to the bath. The mean (± S.D.) amplitudes of peak currents (n=5) in a value relative to the control before, during, and after bath perfusion of nefiracetam at 1, 10, 100, and 1000 nM. B. A bell-shaped dose-response relationship for nefiracetam potentiation of NMDA currents in multipolar neurons (□) (mean ± S.D., n=5), and the absence of nefiracetam potentiation in bipolar neurons (▯) (mean ± S.D., n=4). In bipolar neurons, the S.D. is smaller than the size of the symbol.

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Fig. 3. A. Dose-response relationships of currents induced by 30 μM NMDA in multipolar neurons during bath perfusion of the control solution (○), 10 nM nefiractam (●), 3 μM glycine (▲), and 3 μM glycine plus 10 nM nefiracetam (△). Holding potential –70 mV. Current amplitude was normalized to the maximum control current (mean ± S.D., n=4). B. Peak current amplitudes evoked by 3-1000 μM NMDA in a value relative to the control during perfusion with 10 nM nefiracetam (mean ± S.D., n=4).

Fig. 4. Nefiracetam prevents glycine-induced potentiation of NMDA currents in multipolar neurons. Current were induced by 300 μM NMDA, and 3 μM glycine and 10-10000 nM nefiracetam were applied to the bath. Holding potential –70 mV. A. An example of a series of current records. B. Mean ± S.D. (n=4) of current amplitude relative to the control without glycine/nefiracetam. Glycine-induced potentiation of currents was prevented by nefiracetam at 1000-10000 nM.

Fig. 5. Nefiracetam-glycine interactions in multipolar neurons. A. Dose-response relationships for potentiation of NMDA (30 μM)-induced currents caused by U-tube co-applications of glycine without (□) and with (■) bath applications of 10 nM nefiracetam. Holding potential –70 mV; mean ± S.D.; n = 5. B. Dose-response relationships for potentiation of NMDA (30 μM)-induced currents caused by bath applications of glycine without (□) and with (■) bath applications of 10 nM nefiracetam. Holding potential –70 mV; mean ± S.D.; n = 4.

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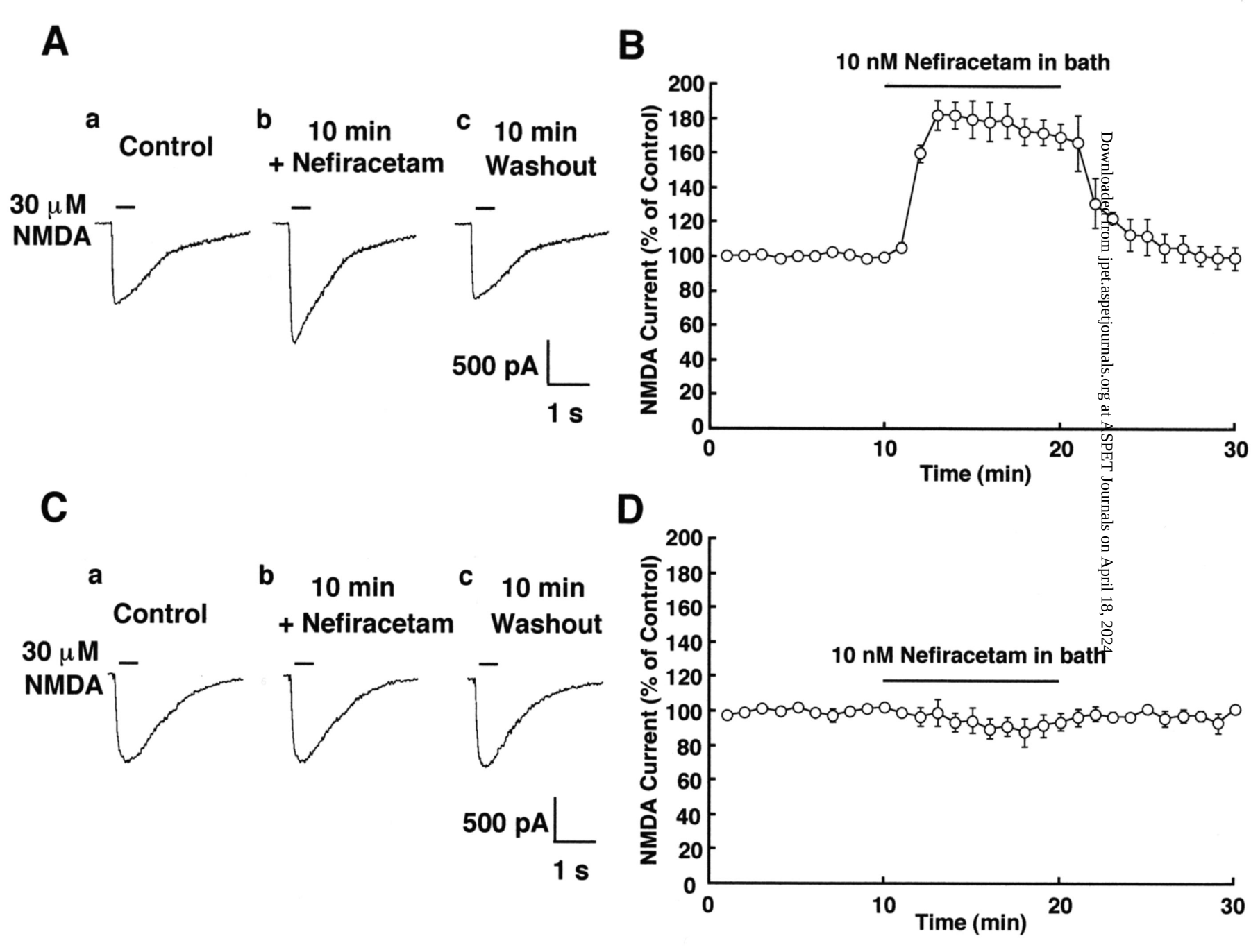
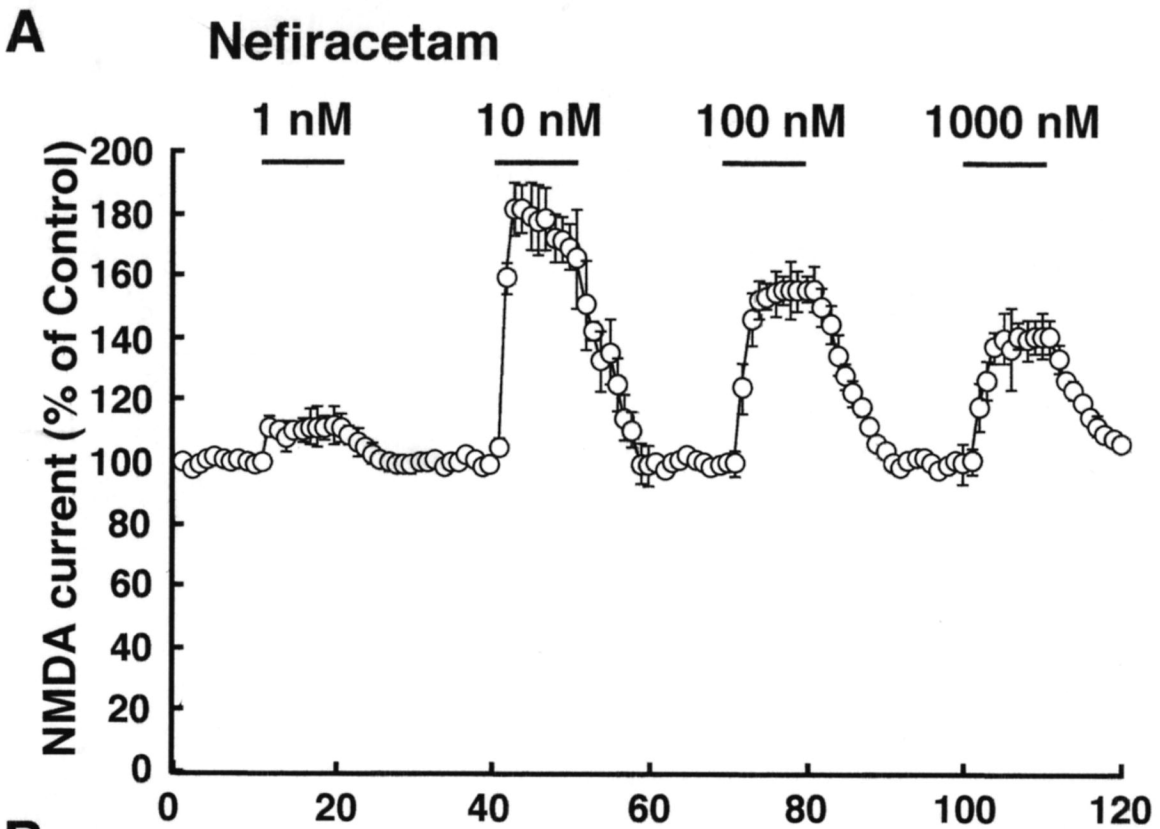
Fig. 6. 7-Chlorokynurenic acid (7-ClKN), a glycine site blocker, prevents nefiracetam potentiation of currents evoked by 30 μM NMDA. A. An example of a series of experiments showing suppression of NMDA current by 1 μM 7-ClKN. B. Time course of changes in NMDA current amplitude during bath application of 300 nM and 1 μM 7-ClKN, 1 μM 7-ClKN plus 10 nM nefiracetam, and 10 nM nefiracetam alone. Letters a-g corresponds to those in A.

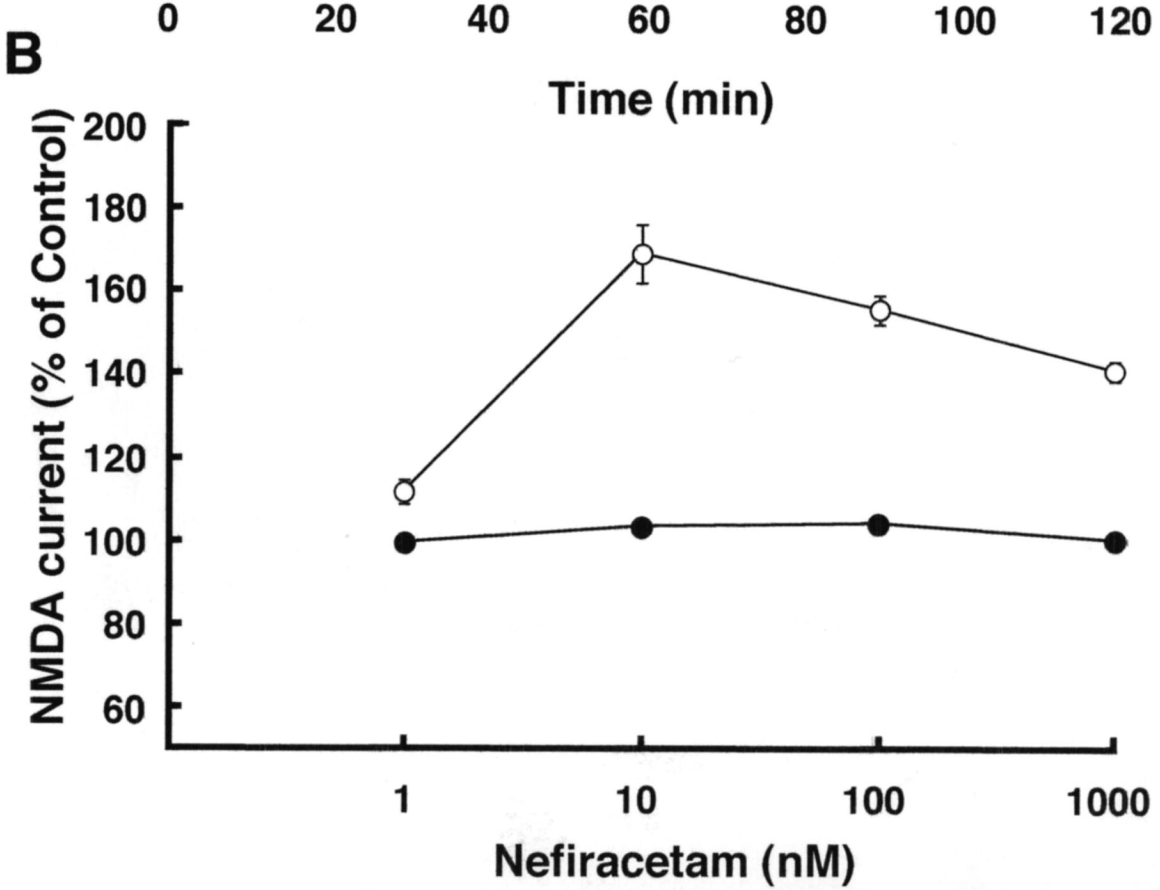
Fig. 7. Nefiracetam 10 nM shifts the dose-response relationship for 7-chlorokynurenic acid inhibition of currents induced by 30 μM NMDA in the direction of higher concentration. Multipolar neurons IC_{50}s: 295.6 ± 31.1 nM in control (n=4); 467.6 ± 24.9 nM in nefiracetam (n=4). 7-ClKN was applied both to the bath and via U-tube, and nefiracetam was applied to the bath. Glycine was added to the external solution at a concentration of 10 nM.

Fig. 8. Effects of bath perfusion of 10 nM nefiracetam on currents evoked by 30 μM AMPA (A and B, n=4) and 30 μM kainate (C and D, n=4), in multipolar neurons. Holding potential –70 mV. Neither AMPA-induced currents nor kainate-induced currents were significantly affected by 10 nM nefiracetam.



Moriguchi et al. Fig. 1





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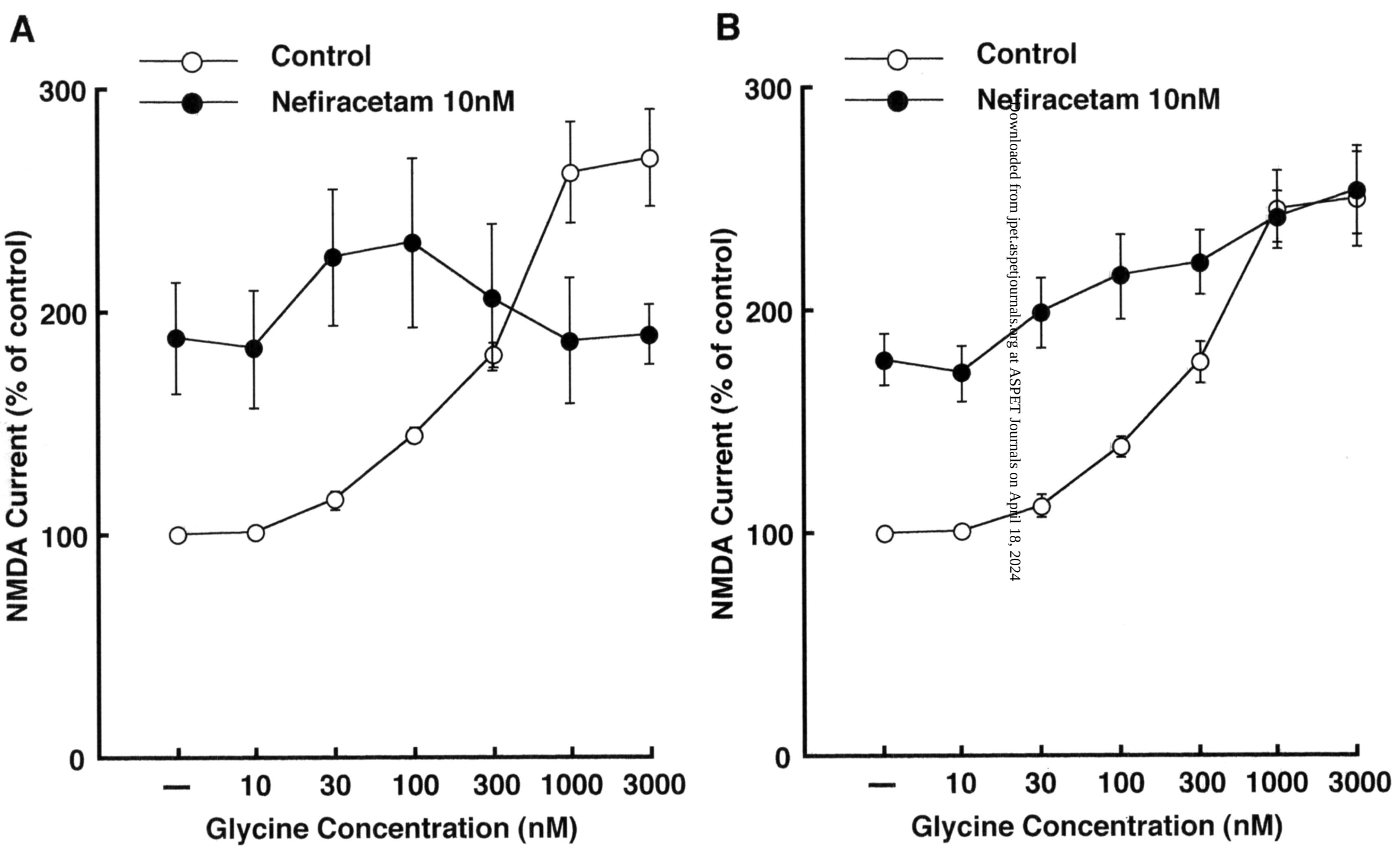

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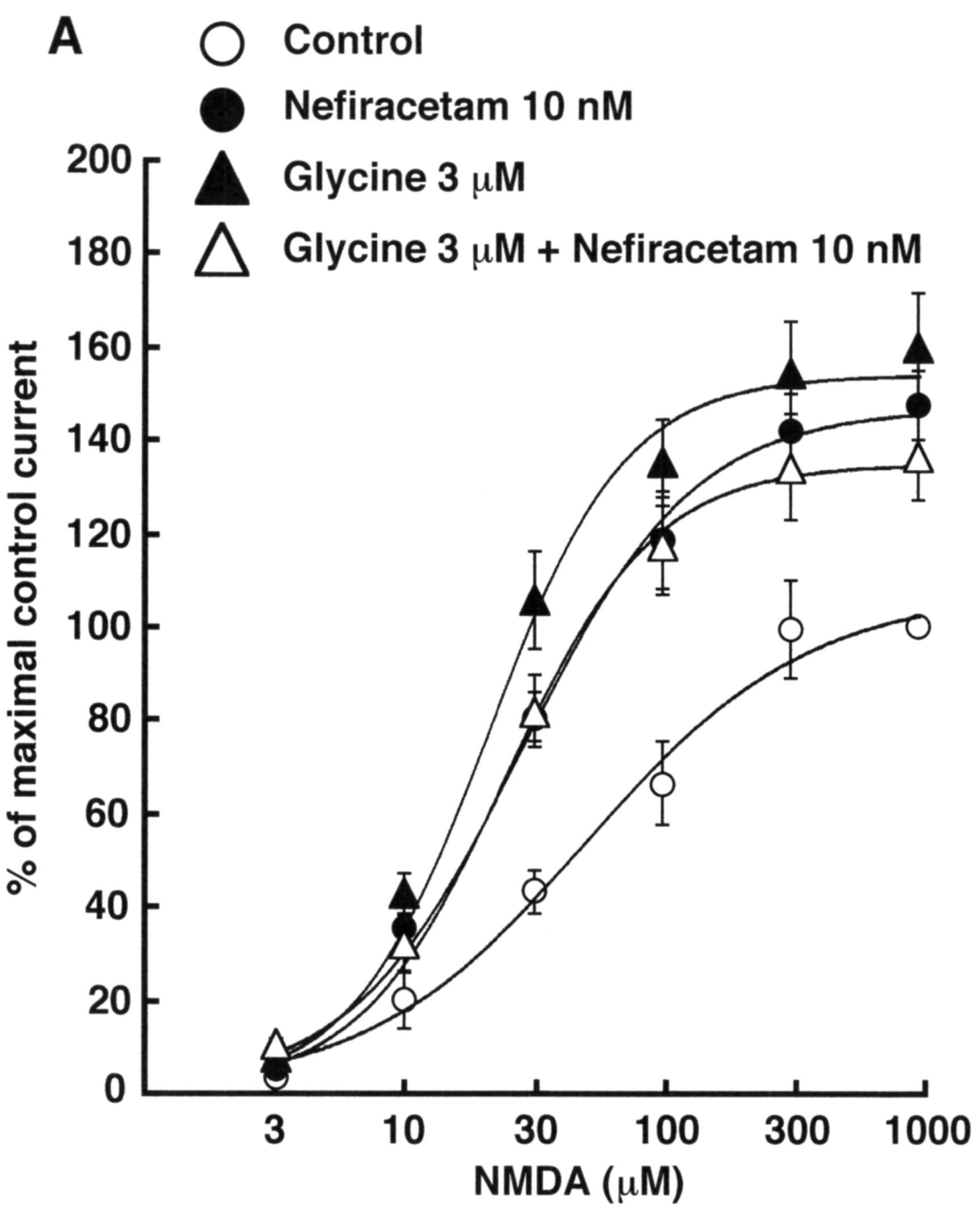
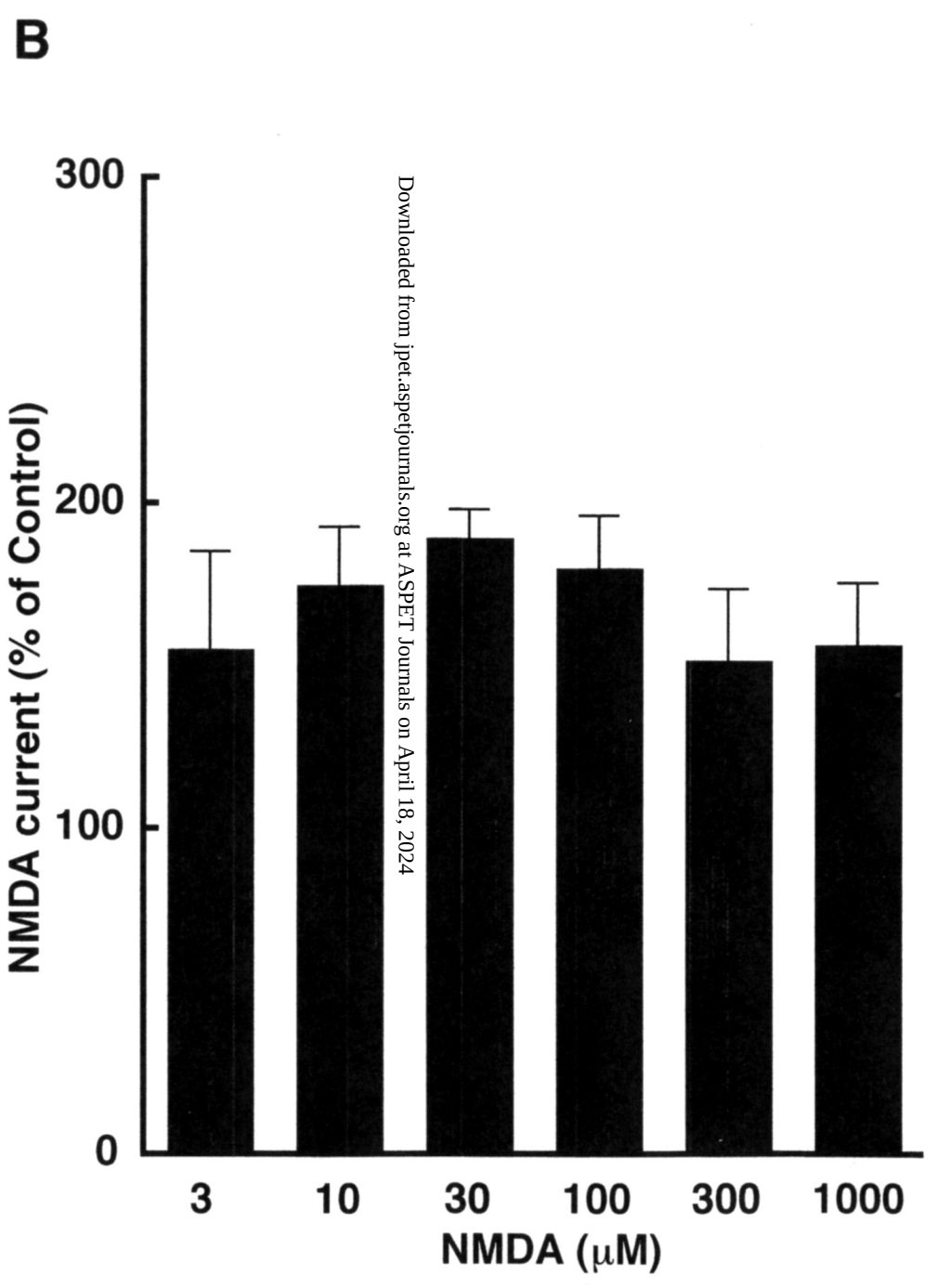
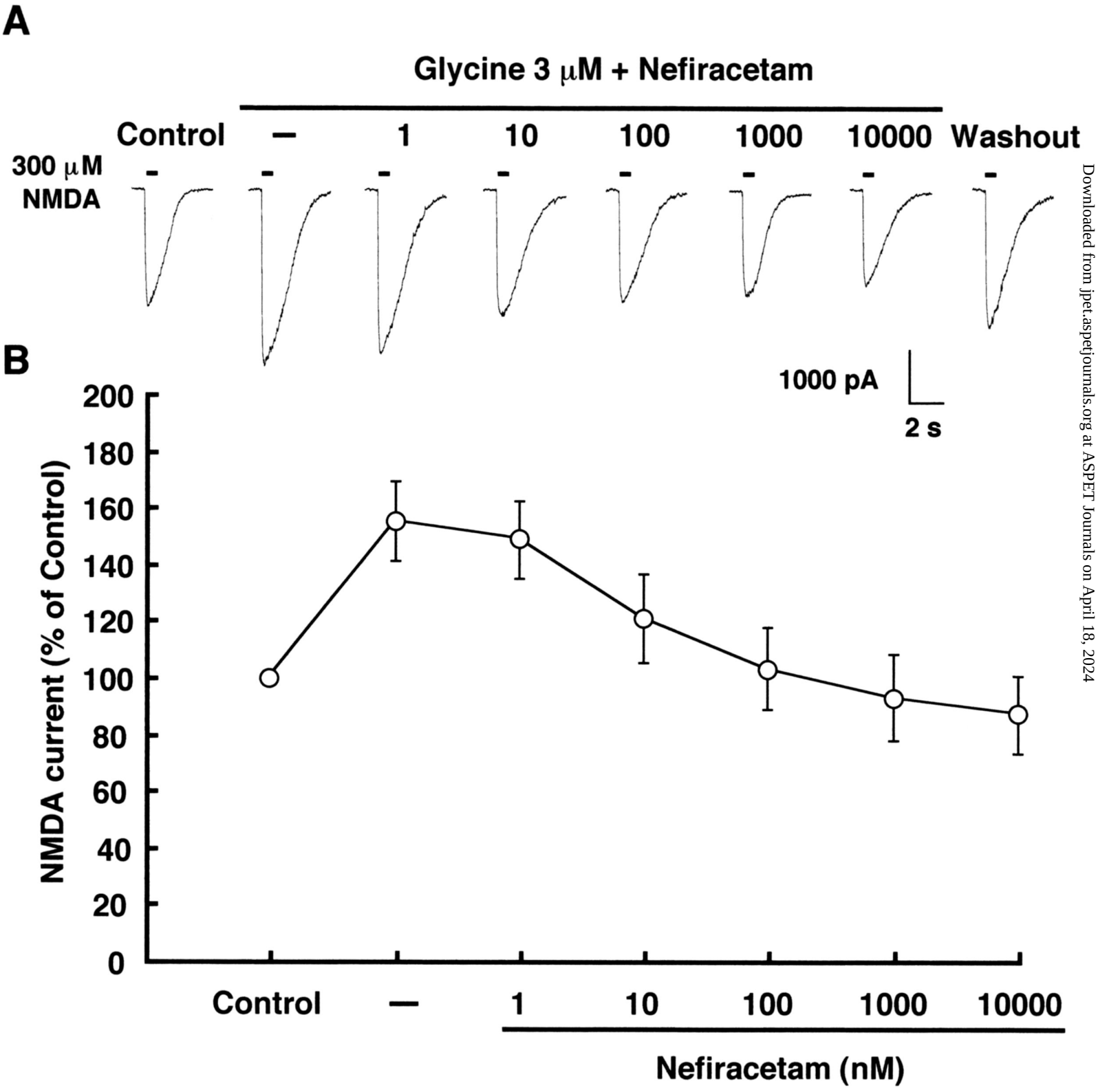




Moriguchi et al. Fig. 3



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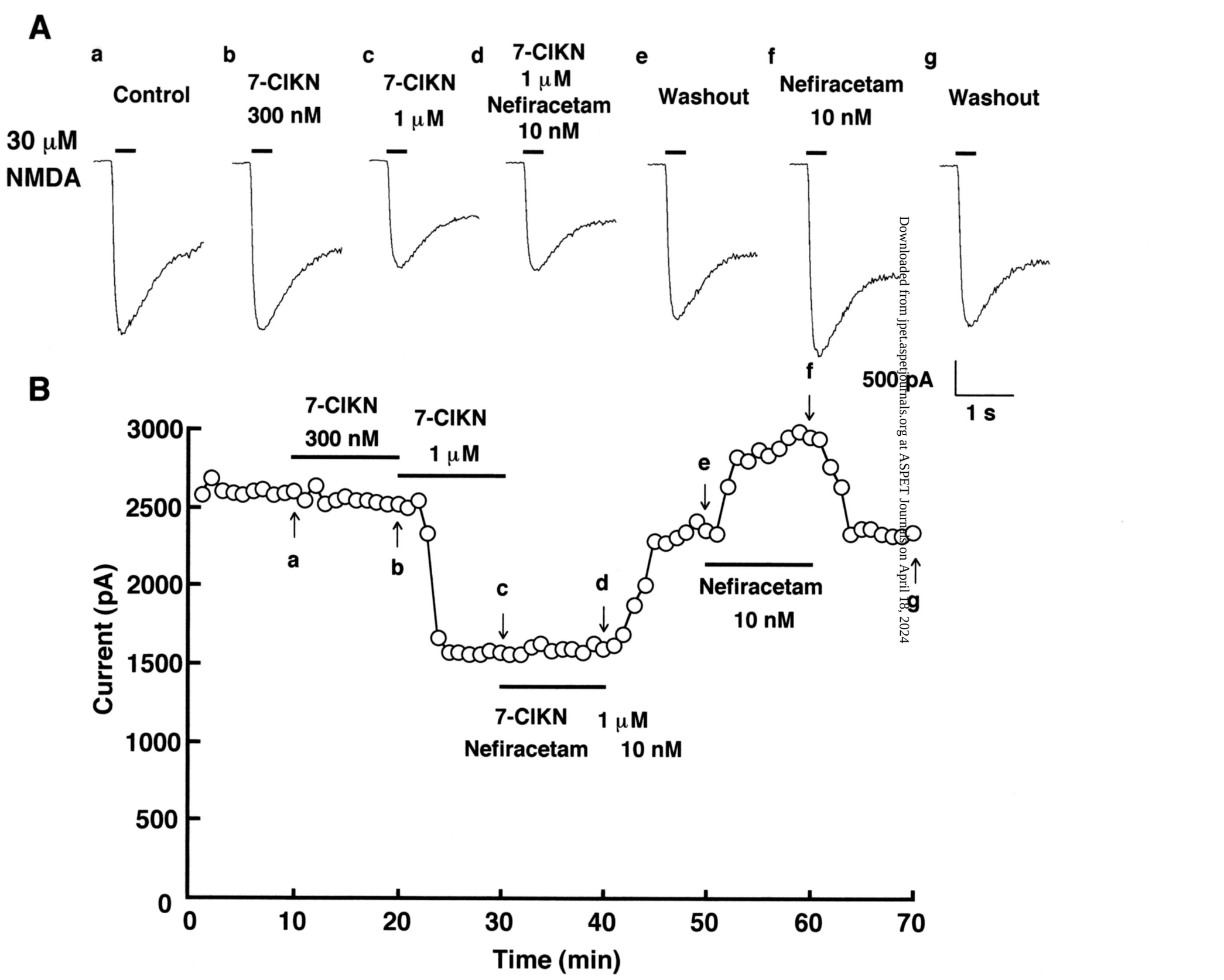

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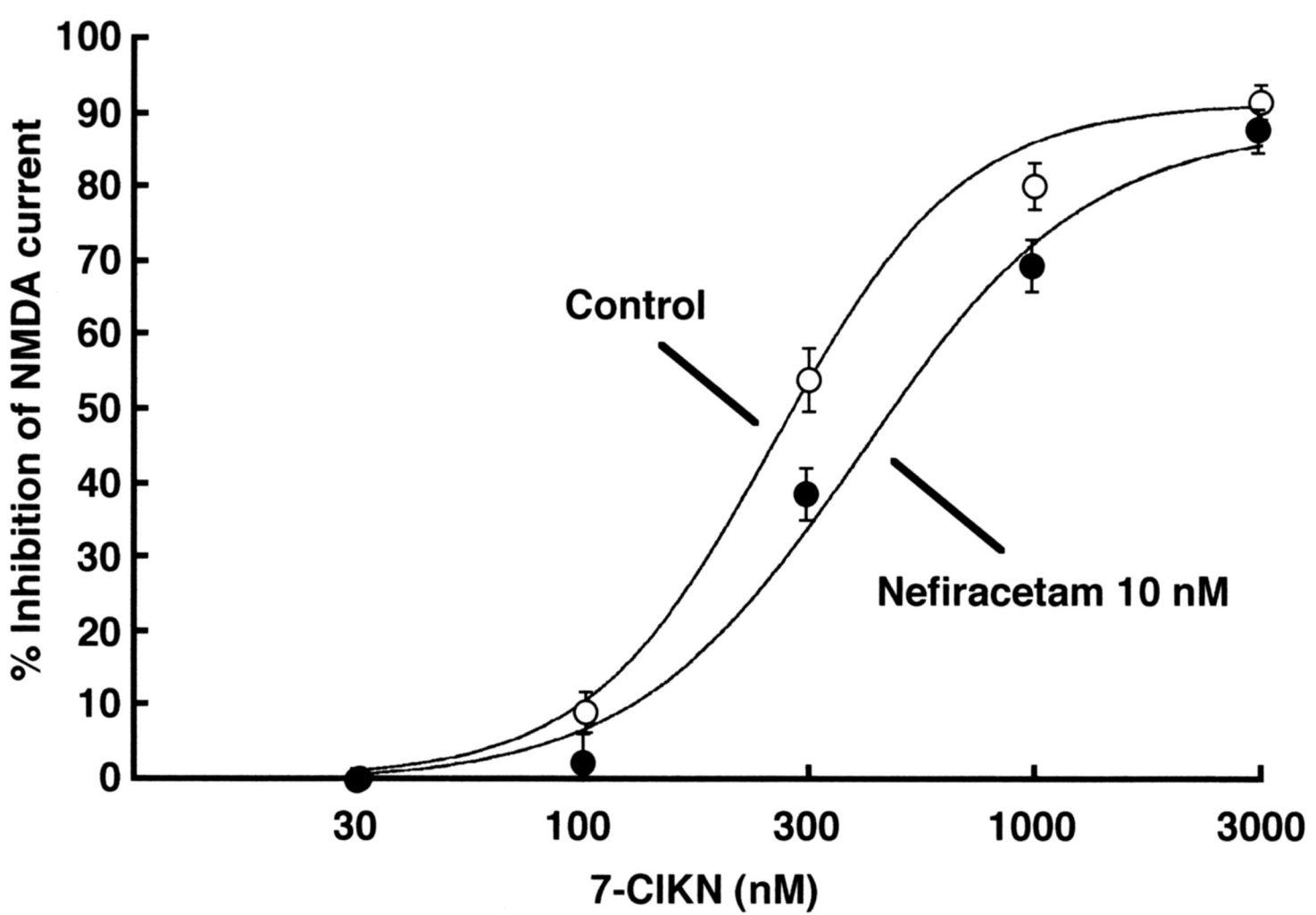

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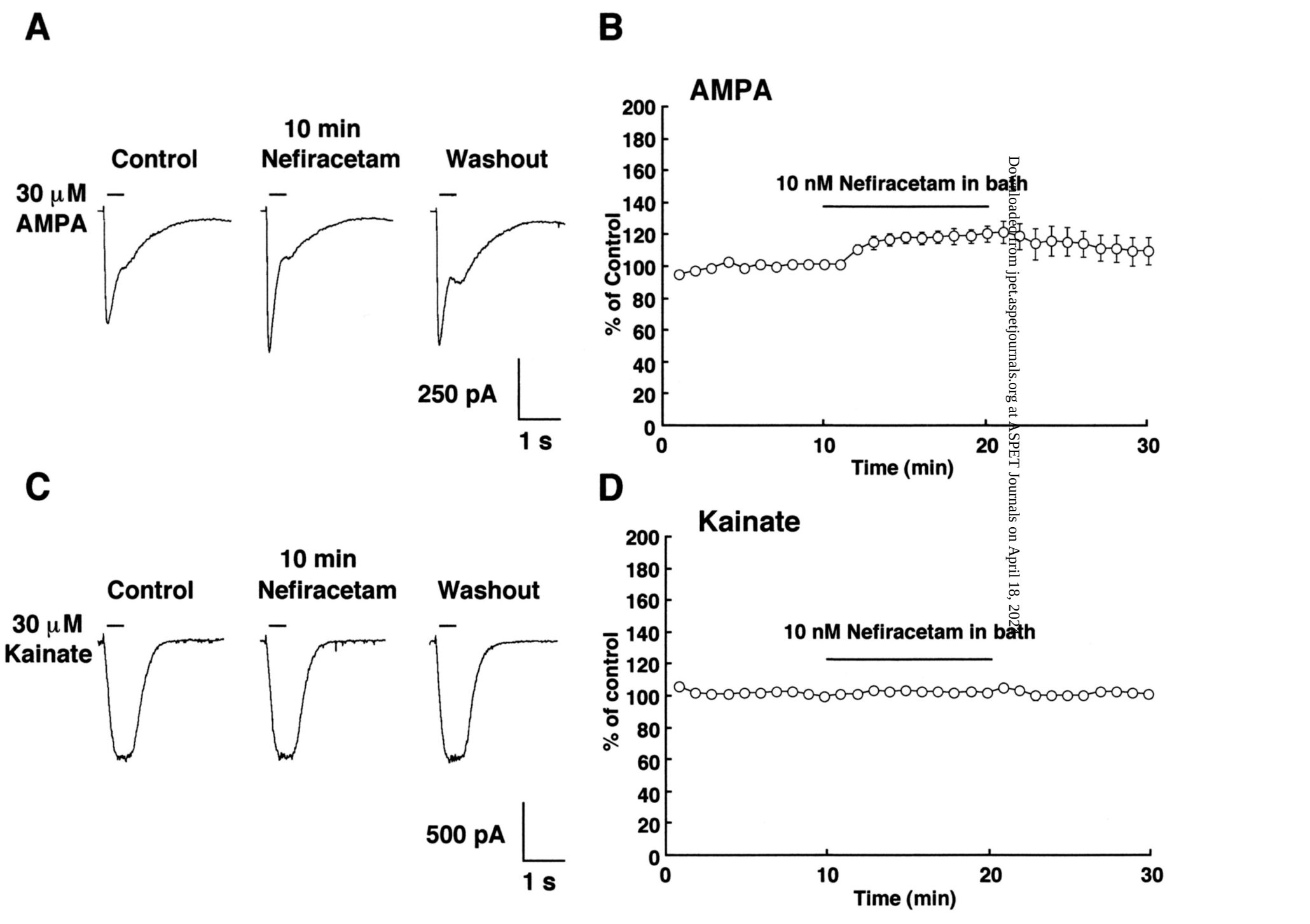

Moriguchi et al. Fig. 8