

Nebracetam (WEB 1881FU) Prevents *N*-Methyl-D-Aspartate Receptor-Mediated Neurotoxicity in Rat Striatal Slices

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ABSTRACT—The effects of nebracetam were investigated on *N*-methyl-D-aspartate (NMDA) receptor- and voltage-operated Ca^{2+} channels (VOCC)-mediated neural dysfunction by directly monitoring the real-time dynamics of dopamine released from rat striatal slices. Nebracetam (10^{-5} and 10^{-4} M) completely protected against striatal dopaminergic impairment induced by L-glutamate and NMDA, respectively. BAY K-8644-evoked striatal dysfunction was not blocked by nebracetam (10^{-4} M). Therefore, nebracetam seems to produce a neuroprotective action by interacting, at least in part, with NMDA receptor-operated Ca^{2+} channels.

Keywords: Nebracetam, Neuroprotective action, Striatal slices (rat)

It is widely accepted that brain damage is associated with neuronal Ca^{2+} overload caused by an excessive influx of extracellular Ca^{2+} (1). An inappropriate Ca^{2+} entry in an etiological event of neuronal injury occurs largely through Ca^{2+} channels gated by the *N*-methyl-D-aspartate (NMDA) receptor, a subtype of glutamate receptor, but also through voltage-operated Ca^{2+} channels (VOCC), $\text{Na}^+/\text{Ca}^{2+}$ exchangers and membrane leakage (1). We have shown that either high K^+ or endothelin participates in the development of hypoglycemia- or hypoglycemia/hypoxia-induced brain damage through the VOCC in an in vitro model by directly monitoring the real-time dynamics of dopamine (DA) released from striatal slices (2, 3). The striatum has been known to be highly vulnerable to hypoxia/ischemia (4) and contains NMDA receptors and 1,4-dihydropyridine binding sites labeling VOCC with a moderate density (5, 6). Therefore, an in vitro model of brain damage we designed may be useful for determining the development of brain damage through receptor-operated Ca^{2+} channels as well as VOCC.

Nebracetam (WEB 1881FU, 4-aminomethyl-1-benzylpyrrolidine-2-one-hemifumarate) has been recently proposed to have a neuroprotective action and cognitive enhancing effect being characteristic of a nootropic

drug (3, 7, 8). The compound protected against hypoglycemia/hypoxia-induced striatal damage (3) and improved the disruption of spatial cognition evoked by scopolamine and Δ^9 -tetrahydrocannabinol (7). The compound also protected against the destruction of hippocampal cholinergic neurons as well as memory impairment induced by ethylcholine aziridinium ion (AF 64A), a neurotoxic choline analog (8). Thus, we report here that glutamate and NMDA as well as high K^+ and BAY K-8644 participate in the development of neuronal dysfunction, and also the neuroprotective action of nebracetam was examined on NMDA receptor-mediated and VOCC-mediated neuronal dysfunction.

Male Sprague-Dawley rats, aged 7–9 weeks and weighing 200–250 g, were decapitated; and then the brains quickly were removed and cut coronally at the level of the anterior commissura. The 450- μm -thick striatal slices were prepared with a McIlwain tissue chopper and mounted in the perfusion apparatus. The perfusion apparatus was fundamentally composed of a tissue perfusion component and an electrochemical detector (ECD) monitoring component, as previously described (2). In brief, the slice sandwiched between two membrane filters (3- μm pore size; Nuclepore, CA, U.S.A.) was placed in a chamber and perfused with oxygenated (95% O_2 /5% CO_2) Krebs-Ringer bicarbonate solution at pH 7.4, of the following composition: 118.0

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mM NaCl, 4.7 mM KCl, 1.3 mM CaCl_2 , 1.2 mM MgCl_2 , 1.0 mM NaH_2PO_4 , 25.0 mM NaHCO_3 and 11.0 mM D-glucose, at a flow rate of 500 $\mu\text{l}/\text{min}$ at 37°C. The perfusate from the tissue chamber was directly introduced into the ECD monitoring component (LC-4B and TL-5, Bioanalytical system, IN, USA), setting the electrode potential at +0.45 V versus Ag/AgCl reference electrode, to detect oxidizable substances released from the striatal slices.

The experiments were started after a 60-min superfusion. The experimental procedures are diagrammed in Fig. 1 (2). Tissues were stimulated 3 times (S1–S3) at 20-min intervals by application of KCl (40 mM). The response to the 3rd stimulation (S3) served as the standard for each set of striatal slices. Thereafter, the tissue was perfused with Krebs-Ringer solution containing sucrose as the substitute for D-glucose (D-G-free Ringer), bubbled with 95% $\text{N}_2/5\%$ CO_2 (hypoglycemia/hypoxia). Following termination of hypoglycemia/hypoxia, the test stimulation with KCl (40 mM) (S4–S6) was carried out 3 times at 20-min intervals under reperfusion with normal Ringer solution. Under the condition of hypoglycemia/hypoxia, the tissue was treated twice at 5-min intervals with KCl (5×10^{-2} M) or treated 3 times at 5 min intervals with L-glutamate hydrochloride (10^{-2} M), NMDA (10^{-4} M) or 1,4-dihydro-2,6-dimethyl-5-nitro-4-[2-(trifluoromethyl)phenyl]-3-pyridinecarboxylic acid methyl ester (BAY K-8644, 5×10^{-5} M) (Research Biochemicals, Inc., MA, USA) (Fig. 1B). L-Glutamate hydrochloride (Research Biochemicals, Inc., MA, USA) and NMDA (Sigma, MD, USA) were dissolved in D-G free Ringer solution which did not contain Mg^{2+} (D-G-free/ Mg^{2+} -free Ringer) to avoid the blockade of NMDA receptor-operated channels by Mg^{2+} . Nebracetam (Nippon Boehringer Ingelheim, Hyogo, Japan) dissolved in D-G-free Ringer or D-G-free/ Mg^{2+} -free Ringer was given during the 20 min period of hypoglycemia/hypoxia (Fig. 1B).

DA release was expressed as the relative response, that is, the ratio of the peak amplitude of the current-time curve in the stimulation-evoked response to that in the standard response to S3 (Fig. 1). In some cases, the concentrations of catecholamines in each individual response were determined by collecting the corresponding aliquot of the perfusate, using high performance liquid chromatography with an ECD (9). There was a high correlation between the magnitude of the output current from the ECD and the amount of released DA in each response to the stimuli, although 3,4-dihydroxyphenylacetic acid also contributed to the output current (3, 10). Therefore, the current-time curve can be interpreted as an estimated real-time dynamics of DA release from the striatal slices. The influences of various

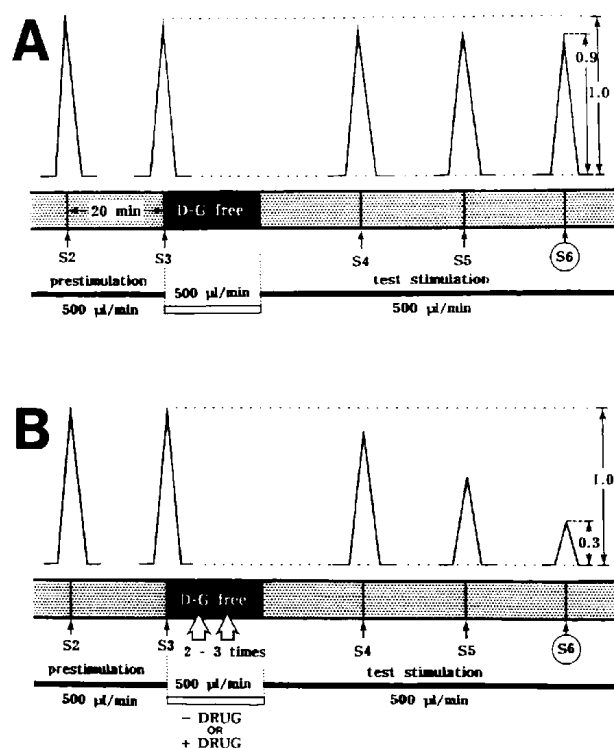


Fig. 1. Diagram showing the experimental procedure. A: tissues were not exposed to any reagents such as L-glutamate, NMDA, BAY K-8644 and 50 mM K^+ during the 20 min of hypoxia/hypoglycemia (control experiment). B: drug experiment. – and +: without and with nebracetam, respectively. Numerals (0.9 and 0.3) on the right side of panels A and B, respectively, show relative response (S6/S3). ■: D-glucose free + 95% $\text{N}_2/5\%$ CO_2 , ↑: various reagents (1 min), †: 40 mM high K^+ (0.5 min).

treatments during the 20-min period of hypoglycemia/hypoxia were indicated as a percentage of the relative response to S6 in the control experiment in which no treatment was carried out during the 20-min period (Fig. 2). Data were expressed as the mean \pm S.E. Statistical analyses were accomplished with Duncan's test for individual comparisons following one-way analysis of variance.

When striatal slices were not exposed to various reagents during the 20-min period of hypoglycemia/hypoxia, the relative response to S6 was 0.85 ± 0.06 of that to S3 ($n = 10$). An inhibition of striatal response to test stimulation (S4–S6) was observed after the pulse treatment with various reagents during the hypoglycemia/hypoxia condition (Fig. 2). In preliminary experiments, we determined the concentrations and number of applications of these reagents necessary to produce almost the same degree of inhibition (40–50% of control). The striatal responses to S6 were $52.2 \pm 4.2\%$, $56.8 \pm 2.9\%$, $61.7 \pm 3.0\%$ and $53.2 \pm 6.0\%$ of the control (no treatment during hypoglycemia/

hypoxia) after the treatment with L-glutamate (10^{-2} M), NMDA (10^{-4} M), BAY K-8644 (5×10^{-5} M) and KCl (5×10^{-2} M), respectively (Fig. 2). The inhibition was not improved, even if the tissue was reperfused with normal Ringer for up to 2 hr after the termination of the hypoglycemia/hypoxia period.

Nebracetam protected against inhibition of the response to S6 induced by treatment with L-glutamate, in a dose-dependent manner (Fig. 2A). The L-glutamate-induced inhibition was recovered by 42.7 ± 18.0 and $75.6 \pm 5.0\%$ with 10^{-6} M and 10^{-5} M of nebracetam, respectively. Nebracetam also protected against inhibition induced by treatment with NMDA, although the protective effect was 10-fold less potent than that obtained in the case of L-glutamate-induced inhibition (Fig. 2A). The recoveries of NMDA-induced inhibition by nebracetam at 10^{-5} M and 10^{-4} M were $54.1 \pm 14.1\%$ and $92.5 \pm 19.2\%$, respectively. Nebracetam protected against the inhibition induced by high K^+ with relatively weak efficacy (Fig. 2B). The recoveries of high K^+ -induced inhibition by nebracetam at 10^{-5} M and 10^{-4} M were $26.4 \pm 18.3\%$ and $38.6 \pm 16.4\%$, respectively. In contrast, nebracetam at a concentration of up to 10^{-4} M failed to protect against inhibition of the response to S6 induced by treatment with BAY K-8644 (Fig. 2B). Nebracetam was effective in protecting against the inhibition of the response to S6 induced by treatment with various reagents during a 20-min period of hypoglycemia/hypoxia, in the following order: L-glutamate- > NMDA- > high K^+ -induced inhibition.

The present study demonstrated that the treatment with four reagents, L-glutamate, NMDA, BAY K-8644 and KCl during hypoglycemia/hypoxia enhanced the hypoglycemia/hypoxia-induced dopaminergic dysfunction, as observed in the case of treatment with high K^+ and endothelin (2, 3). An inappropriate influx of Ca^{2+} leads to central neuron injury (1). The hypoglycemia-induced dopaminergic dysfunction was not enhanced by treatment with endothelin in the absence of external Ca^{2+} during hypoglycemia (2). Thus, these four reagents appear to enhance the hypoglycemia/hypoxia-induced damage of striatal dopaminergic neurons by increasing intracellular Ca^{2+} concentration, since these four reagents are accepted to activate the Ca^{2+} channels (11). Such dopaminergic dysfunction induced by L-glutamate/NMDA and BAY K-8644/high K^+ may be attributed to increased Ca^{2+} influx through NMDA receptor and VOCC, respectively. In the present study, the stimulation-evoked release of DA was examined as an index of the striatal neuronal dysfunction. In another study, histological examinations have shown that hypoglycemia/hypoxia in the in vitro model used in the present study produced neuronal damage in the

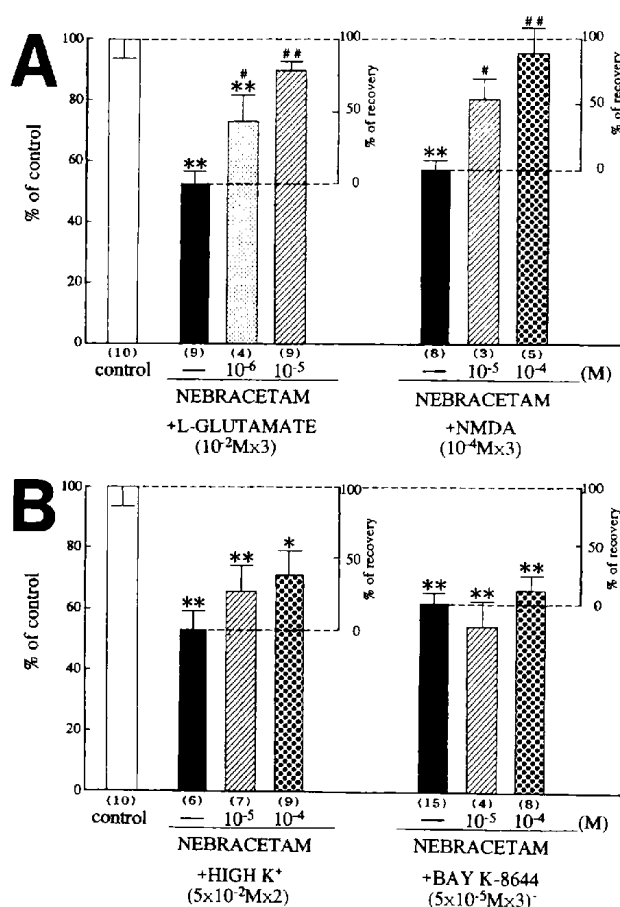


Fig. 2. Effects of nebracetam on dopaminergic impairment induced by L-glutamate (A), NMDA (A), BAY K-8644 (B) and 50 mM K^+ (B) in rat striatal slices. During the 20 min period of hypoxia/hypoglycemia, tissues were exposed to 10^{-2} M of L-glutamate 3 times for 1 min each at 5-min intervals (L-glutamate, 10^{-2} M \times 3), NMDA (10^{-4} M \times 3), BAY K-8644 (5×10^{-5} M \times 3) and KCl (5×10^{-2} M \times 2). Values represent the mean \pm S.E. Numeral in parentheses at the bottom of each column indicates the No. of experiments. % of control: a percentage of the relative response to S6 in the control experiment. % of recovery was calculated as: (evoked release in the presence of nebracetam - evoked release in the absence of nebracetam) \div (control - evoked release in the absence of nebracetam) \times 100. * P < 0.05, ** P < 0.01; significantly different from the control. * P < 0.05, ** P < 0.01; significant difference between without (-) and with nebracetam.

striatum (3). Therefore, there is possibility that hypoglycemia/hypoxia produces and these four reagents enhance the damage of neurons innervating the striatum, including dopaminergic neurons.

Nebracetam was found to protect effectively against the NMDA receptor-mediated neural dysfunction rather than the VOCC-mediated one. We also showed that this compound protected against the high K^+ -induced dopaminergic dysfunction, as was shown previously (3), although its protective effect against high

K⁺-induced dysfunction was much less potent than that against L-glutamate/NMDA-induced dysfunction. In this study, the recovery of L-glutamate/NMDA-induced inhibition by nebracetam was almost complete; however, the recovery of high K⁺-induced inhibition was only partial. Furthermore, nebracetam failed to protect against the BAY K-8644-induced dopaminergic dysfunction. The NMDA antagonists, MK801 and D-amino-5-phosphonopentanoate, provided protection against the dysfunction of striatal neurons induced by NMDA and L-glutamate (12), while the Ca²⁺ antagonist nifedipine was much more effective at improving neuronal dysfunction induced by BAY K-8644 and high K⁺ (13). Thus, nebracetam seems to possess the properties of an NMDA antagonist rather than a Ca²⁺ antagonist. The predominant route of lethal Ca²⁺ entry in the case of hypoglycemic/ischemic brain damage is known to be a channel gated by the NMDA receptor (1). Therefore, nebracetam may be useful for the treatment of hypoxia/ischemia-induced brain damage. In conclusion, nebracetam produces a neuroprotective action on hypoxic/ischemic striatal impairment by interacting, at least in part, with NMDA receptor-operated Ca²⁺ channels, although many questions still remain as to the precise molecular mechanisms by which nebracetam modulates NMDA receptor-operated Ca²⁺ channels.

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