Effects of TND1128 (a 5-deazaflavin derivative), with self-redox ability, as a mitochondria activator on the mouse brain slice and its comparison with β-NMN

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Abbreviations

Aβ β-amyloid; ACSF. Artificial cerebrospinal fluid; AD.Alzheimer's disease; AM. Acetoxymethyl; AUC. Area under the curve; CA1. Hippocampal CA1 region; $[Ca^{2+}]_{cyt}$. Cytosolic Ca^{2+} concentration; $[Ca^{2+}]_{mit}$. Mitochondrial Ca^{2+} concentration; CTX. Cerebral cortex; ER. Endoplasmic reticulum; ETC. electron transfer chain; MCU. Mitochondrial Ca^{2+} uniporter; NCX. Na^{+}/Ca^{2+} exchanger; NCLX. $Na^{+}/Ca^{2+}/Li^{+}$

exchanger; PD. Parkinson's disease; β-NMN. β-nicotinamide mononucleotide; VOCC. Voltage- operated Ca^{2+} channel; VONC. Voltage operated Na^{+} channel; 5-Dfa. 5-deazaflavin; 80K. 80 mM KCl ACSF;

Abstract

We have no definitive treatment for dementia characterized by prolonged neuronal death due to the enormous accumulation of foreign matter, such as β-amyloid. Since Alzheimer's type dementia develops slowly, we may be able to delay the onset and improve neuronal dysfunction by enhancing the energy metabolism of individual neurons. TND1128, a derivative of 5-deazaflavin, is a chemical known to have an efficient self-redox ability. We expected TND1128 as an activator for mitochondrial energy synthesis. We used brain slices prepared from mice 22 ± 2 hours pretreated with TND1128 or β-NMN. We measured Ca^{2+} concentrations in the cytoplasm ($[Ca^{2+}]_{cyt}$) and mitochondria ($[Ca^{2+}]_{mit}$) by using fluorescence Ca^{2+} indicators, Fura-4F, and X-Rhod-1, respectively, and examined the protective effects of drugs on $[Ca^{2+}]_{cyt}$ and $[Ca^{2+}]_{mit}$ overloading by repeating 80K exposure. TND1128 (0.01, 0.1, and 1 mg/kg s.c.) mitigates the dynamics of both $[Ca^{2+}]_{cyt}$ and $[Ca^{2+}]_{mit}$ in a dose-dependent manner. β-NMN (10, 30, and 100mg/kg s.c.) also showed significant dose-dependent mitigating effects on $[Ca^{2+}]_{cyt}$, but the effect on the $[Ca^{2+}]_{mit}$ dynamics was insignificant. We confirmed the mitochondria-activating effect of TND1128 by the present study. We expect TND1128 as a drug that rescues deteriorating neurons with aging or disease. **(193 words**)

Key word: Deazaflavin; Ca^{2+} imaging; Mitochondria; Ca^{2+} overload;
β-NMN (nicotinamide mononucleotide**)**

1 Introduction

Unlike cells in other organs, each neuron in our brain is built into specific positions in the neural network of the brain and continues to function throughout our lives. Every neuronal cell works vigorously every moment, receiving tremendous input from other neurons, sending out the information as electrical signals, and releasing neurotransmitters. These processes are highly energy dependent. Glucose, as the sole source of energy in the brain, is delivered to all neurons in a highly developed cerebrovascular system in association with astrocytes.[1]

In recent years, according to the increase in the aging population, the incidence of diseases associated with neuronal degeneration, such as cerebral hemorrhage or infarction, Alzheimer's disease (AD), and Parkinson's disease (PD) has been increasing. Unfortunately, there is no definitive treatment for these diseases to date. AD is a type of dementia characterized by prolonged neuronal death due to the enormous accumulation of β-amyloid (Aβ). This cell death is thought to be due to the toxicity of oligomeric Aβ.[2] However, the progression of neuronal cell death is too slow to ascribe it to the toxicity of these molecules. Recently some researchers have indicated that damage to the brain vasculature is a critical cause of neurodegenerative disorders, such as AD, PD, and Huntington's disease.[3-7] Even though brain vasculature is normal, enormous accumulation of abnormal molecule, such as β-amyloid physically interferes with the brain blood flow. Since this type of disease develops slowly, even if we cannot remove these abnormal molecules, we may be able to delay the onset and improve symptoms by enhancing the energy metabolism of individual neurons.

Imai et al. (2010) demonstrated the antiaging effects of β-NMN (nicotinamide mononucleotide; Fig.1 a), which will activate *Sirtuin* genes, the so-called "longevity genes."[8] β-NMN is proven to have an effective redox activity and is a precursor of NAD^+ and $NADP^+$, cofactors for the redox process of energy production, by conjugating with adenosine diphosphate (ADP) inside cells.[9,10] Mills et al. (2016) report that the production of tissue NAD^+ in mice fed with foods containing β-NMN increases and suppresses the physiological decline in individuals due to aging.[11] Since NAD^+ is the most critical molecule for participating in the energy production as a redox molecule involved in both anaerobic and aerobic glycolysis, we expected that β-NMN as an activator for deteriorating neuronal cell. Some studies reported the effectiveness of β-NMN for improving the cognitive function in acute AD model mice.[12,13]

In the present study, we searched for novel substances that can incorporate energy production as a cofactor in the electron transfer chain. We encountered a

compound, a 5-deazaflavin derivative (10-ethyl-3-methylpyrimido[4,5-*b*]quinoline-2,4(3*H*,10*H*)-dione; TND1128; Fig.1b), which is one of the flavin analogues synthesized to obtain compounds exhibited an efficient redox function as a NAD and FAD.[14-15] Nagamatsu and Akaike (2019) revealed the activity of TND1128 as a coenzyme factor for activation of ATP production in cultured astrocytes.[16] Recently, Katsurabayashi et al. (2021) reported that TND1128 promoted prominent branching of axons and dendrites in cultured neuronal cells.[17]

In the present study, we examined the protective effect of TND1128 from severe depolarization-induced Ca^{2+} overloading in the cytosol and mitochondria in mouse brain slice preparations to know its potential as the energy production activator and compared it with β-NMN.

2. Materials and Methods

2.1 Animal experiment ethics and animal care

All animal experiments in this study were approved by the Institutional Animal Care and Use Committee of Tokyo Medical University (approval number, R1-0132) and were performed in accordance with institutional and national guidelines for animal experimentation.

Male adult C57BL/6N mice (4-weeks old purchased from CLEA Japan, Inc. Tokyo, Japan) were housed in a multi-chamber animal housing system (LP-30LED 8 AR: Nippon Medical & Chemical Instruments Co., Ltd., Osaka, Japan). Each chamber was maintained on a 12 h:12 h light-dark cycle, with the light cycle starting at 09:00, under constant temperature and humidity (23 ± 1ºC and 50% - 60%). Mice were housed in a group (maximum four in a cage) and had *ad libitum* access to food and water. Since several studies have shown that the activities of brain mitochondria are sex-linked, we used only male mice in the present study.[18,19]

2.2 Adjustment and administration of test drugs:

We prepared data for the present study from 38 mice (5 – 18 weeks old), eight as a control group and 30 for testing the effects of TND1128 and β-NMN. We selected mice based on the date of purchase so that the particular drug or its dose was not skewed toward a particular age. We administered each drug dose by a single subcutaneous injection (TND1128 or β-NMN) to a mouse 22 ± 2 hours before preparing brain slices. We decided the time and the way for pre-administration drugs according to the report by Long et al. (2015), who administered β-NMN (100 mg/kg, s.c.) every other day for 28 days to prove the improving effect on mitochondrial oxygen consumption in the brain of

AD model mice.[12]

We prepared the injection solution of β-NMN (1 mg/ml, 3 mg/ml, or 10 mg/ml) using pure water. Since TND 1128 is highly hydrophobic, we prepared a stock solution (5 mg/ml) by triturating 5 mg of TND1128 crystal using an agate mortar and pestle in 1 ml of pure ethanol. We diluted this stock solution 50 times with pure water to make the injection solution of the maximum dose (0.1 mg/ml for 1 mg/kg) and diluted it ten times and 100 times (for 0. 1 mg/kg and 0.01 mg/kg). Although the vehicle of the highest dose of TND1128 includes 2 % ethanol (0.2 g/kg), since this dose was less than one-tenth to cause noticeable pharmacological effects[20] and our experiment started 22 hrs after the drug administration, we did not worry about the effects of this amount of ethanol. Thus we used brain slices prepared from mice treated with 0.1 ml pure water/10 g as the control.

2.3 Preparation of mouse brain slices

We prepared brain slice preparations according to the method descried in the previous paper.[21] We isolated whole brain from drug pretreated or control mice under deep anesthesia with isoflurane (Forane®,Abbott Japan , Tokyo), and prepared four horizontal whole-brain slices (thickness: 300 μm) using a tissue slicer (DTK-1000, Dosaka EM Co. Ltd., Kyoto, Japan). Whole-brain slices were separated into the right and left hemispheres and used randomly as hemi-brain preparations. Preparations were maintained for at least 30 min in a large volume of oxygenized (95% O_2 and 5% CO_2) ACSF at room temperature (25 ± 1ºC).

2.4 Compositions of experimental media

Normal ACSF contains the following (concentrations in mM): NaCl (124.0), $NaHCO_3$ (26.0), KCl (2.5), $CaCl_2$ (2.0), $MgCl_2$ (1.0), NaH_2PO_4 (1.25), and D-glucose (10.0). The composition of 80K-ACSF is (concentrations in mM): NaCl (46.5), $NaHCO_3$ (26.0), KCl (80.0), $CaCl_2$ (2.0), $MgCl_2$ (1.0), NaH_2PO_4 (1.25), and D-glucose (10.0). These solutions were oxygenized with 95% O_2 and 5% CO_2. The temperature of the medium was maintained at 32 ± 1ºC using a self-made temperature control unit that had been previously tested for reliability.[21,22]

2.5 Loading with two different fluorescent Ca^{2+} indicators

We used two pieces of hemi-brain slices from a mouse treated with each drug or vehicle, one for cytosolic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) and the other for mitochondrial Ca^{2+} concentration ($[Ca^{2+}]_{mit}$) measurement. We incubated slice preparations in constantly oxygenized normal ACSF containing 5 μM of Fura-4F/AM (for $[Ca^{2+}]_{cyt}$) or X-Rhod-

1/AM (for $[Ca^{2+}]_{mit}$) for 45 min at room temperature (25 ± 1°C). We transferred these preparations into fresh ACSF and incubated them for 30 min to enzymatically digest to free forms of both Ca^{2+} indicators. We measured the Fura 4F (Kd[Ca^{2+}] = 770 nM) fluorescence (>500 nm) induced by 360 nm (F360) excitation, which is insensitive to Ca^{2+}, and 380 nm (F380) which decreases with an increase in Ca^{2+}.[23] We obtained the cytosolic Ca^{2+} concentration dynamics as F360/F380. The merit of using the F360 signal is that the signal is available as an indicator of cytosolic volume change.[21] We detected $[Ca^{2+}]_{mit}$ dynamics by X-Rhod-1 (Kd[Ca^{2+}] = 700 nM) as red fluorescence (600 nm) induced by 580 nm excitation (F580).[24,25] X-Rhod-1 is known to remain partially in the cytosol, and the signal of $[Ca^{2+}]_{cyt}$ may overlap that of $[Ca^{2+}]_{mit}$.[26] However, since the X-Rhod-1 signal induced by isotonic 80 mM KCl ACSF (80K) stimulation is high enough to regard it as dominantly due to $[Ca^{2+}]_{mit}$. Thus we accepted the present results using X-Rhod-1 as a critical index of mitochondrial Ca^{2+} dynamics.

2.6 Measurement of Ca^{2+} concentration in cytosol or mitochondria

We placed and fixed a hemi-brain slice preparation loaded with Fura-4F or X-Rhod-1 in a recording chamber locked on the stage of an inverted fluorescence microscope (IX71; Olympus Co., Tokyo, Japan). It was constantly perfused with fresh ACSF (2 mL/min) using a microtube pump (MP-32; Tokyo Rikakikai Co., Ltd., Tokyo, Japan). We obtained two-dimensional fluorescence images of the preparation according to the same procedure reported previously (Fig.2).[21,22] We obtained the averaged fluorescence intensity induced by 100 msec excitation from regions of interest (arbitrary square consisting of 200 – 300 pixels) on CTX and CA1 every 10 sec for 40 min.

After measurement at 5 min at the resting level, the hemi-brain slices prepared from mice treated with each concentration of the two drugs were exposed to 80K for 5 min and then returned to normal ACSF for 5 min. We repeated this procedure three times, once every 10 min. We normalized the fluorescence responses based on the value obtained at the first administration of 80K and calculated the responses as the area under the curve (AUC), as shown in Supplemental Fig. 1.

2.7 Reagents and chemicals

The following reagents and chemicals were used: TND1128 (10-ethyl-3-methylpyrimido[4,5-*b*]quinoline-2,4(3*H*,10*H*)-dione)(10-ethyl-3-methyl-5-deazaflavin) synthesized by co-author (T.N.)(Fig.1b)(Nagamatsu & Akaike, 2019), β−NMN (Sigma-Aldrich, Japan, Tokyo Lot.SLBT5015, SLB03074V)(Fig.1 a); Fura-4/AM (Invitrogen, Tokyo, Japan, Lot.194899) and X-Rhod-1/AM (Invitrogen Tokyo, Japan, Lot. 1970975).

2.8 Statistical analysis

We used JMP Pro 15 Statistical Discovery (SAS Institute, North Carolina, USA) for data analysis. We used a one-way analysis of variance (ANOVA) to test for differences in means among the control and three groups treated with different doses. When the result of ANOVA becomes significant ($p < 0.05$), we examined the set by the Tukey test. We put asterisks between the groups according to their rates of significance (* $p < 0.05$; ** $p < 0.01$ and *** $p < 0.005$). We examined the homoscedasticity of each data set by Levene's test. When the Levene's test rejects homoscedasticity, we mark the set with a dagger to note its uncertain significance.

3 Results

3.1 Dynamics of intracellular and mitochondrial Ca^{2+} concentration induced by three consecutive administrations of 80K

Fig.2 shows examples of the Ca^{2+} concentration changes in the cytosol ($[Ca^{2+}]_{cyt}$) (**a**) and mitochondria ($[Ca^{2+}]_{mit}$) (**b**) of the mouse CTX and CA1 obtained from the control mouse induced by exposure to 80K.

As shown by the arrow in Fig.2a, the rise of $[Ca^{2+}]_{cyt}$ in both the CTX and CA1 regions following the first application of 80K was slow. The F360 signal of Fura-4F, which is insensitive to the Ca^{2+} concentration, showed small but apparent transient decreases (shown by an asterisk) when applied to 80K, and it automatically recovered before washing (Supplemental Figure 2 a). The recovery of $[Ca^{2+}]_{cyt}$ in both regions seemed to be poor in the first five minutes after replacing 80K with normal ACSF.

Fig.2b shows X-Rhod-1-loaded preparation obtained from the same mouse used in Fig.2a. The $[Ca^{2+}]_{mit}$ dynamics detected by the fluorescence (F580) in CTX induced by the three administrations of 80K was different from that of $[Ca^{2+}]_{cyt}$ (Fig.2a). The first reaction to the 80K challenge in CTX and CA1 appeared sharply.

3.2 Effects of β-NMN on $[Ca^{2+}]_{cyt}$ changes induced by 80K administration

Fig. 3a shows the patterns of $[Ca^{2+}]_{cyt}$ responses to 80K at CTX and CA1 in Fura-4F-loaded slice preparations obtained from control ($n = 8$) and β-NMN-treated mice (pretreated for 22 ± 2 hours with 10, 30, and 100 mg/kg, s.c.; $n = 5$ each). The patterns of $[Ca^{2+}]_{cyt}$ responses to 80K in mice pretreated with β-NMN (10 mg/kg and 30 mg/kg, s.c.) were similar to those of controls, but those obtained from β-NMN (100 mg/kg, s.c.) pretreated mice were different responses. As shown in Fig.3b, we can find a significant effect of β-NMN on the total $[Ca^{2+}]_{cyt}$ (see Supplemental Figure 1) in the CTX region

(F=3.78; $p < 0.05$; $P < 0.05$ by Tukey test). However, there was no significant effect on the CA1 region (F= 2.51; $p > 0.05$).

There was significant dose-dependent decrease in response to the 2nd (F = 4.63; $p < 0.05$) and 3rd challenges (F = 4.12; $p < 0.05$) in CTX. We could not find any dose-dependent variations in the effects on the CA1 region (Fig 3 c right panel).

As a marker of recovery from excess calcium inflow, we determined the rate of recovery by the value obtained as the ratio between the elevated $[Ca^{2+}]_{cyt}$ during 5 min 80K depolarization over 5 minutes of washing (see Supplemental Figure 1). We can detect a significant effect on the 80K-induced $[Ca^{2+}]_{cyt}$ increase in the 100 mg/kg β-NMN treated group (F=4.47, $p< 0.05$) at the first challenge. However, we found the homoscedasticity of the data set was statistically insignificant using Levene's test (F = 8.16; $P < 0.05$; note dagger marks). The recovery rates from 2nd and 3rd challenges were rather stable except for the response observed in CTX treated with 100 mg/kg.

3.3 Effects of β-NMN on $[Ca^{2+}]_{mit}$ changes induced by the administration of 80K

Fig.4a shows mitochondrial Ca^{2+} concentration ($[Ca^{2+}]_{mit}$) detected in X-Rhod-1-loaded brain slices obtained from the same mice pretreated with β-NMN (10, 30, and 100 mg/kg, s.c.) used in the above experiments. As shown in Fig.4b, the total $[Ca^{2+}]_{mit}$ observed during three times 80K stimulation appears to be reduced in a dose-dependent manner. However, there is no statistical significance (CTX, F = 2.20; CA1, F=2.47). As shown in Fig.4c, β-NMN showed no statistical significance on the $[Ca^{2+}]_{mit}$ increase induced by the first and the second 80K stimulation in both CTX and CA1. We can find a tendency to mitigating effects of β-NMN on the $[Ca^{2+}]_{mit}$ induced by the third 80K stimulation induced (CTX F = 2.75; CA1 F = 2.84).

The recovery of $[Ca^{2+}]_{mit}$ by 5-min washing from the increase caused by 2nd and 3rd 80K stimulation was very stable in each preparation at every dose~~, unlike that observed in the $[Ca^{2+}]_{cyt}$ dynamics~~ (Fig.4 d).

3.4 Effects of TND1128 on $[Ca^{2+}]_{cyt}$ changes induced by the administration of 80K

Figure 5a shows the patterns of $[Ca^{2+}]_{cyt}$ responses to 80K of CTX and CA1 in Fura-4F-loaded slice preparations obtained from control (N=8) and TND1128-treated mice (pretreated for 22±2 hours with 0.01, 0.1, or 1 mg/kg, s.c.; n = 5 each). As shown in Figure 5a, at each dose, TND1128-treated preparations showed similar patterns (delayed increase) of response to the administration of 80K to those observed in the preparations obtained from100 mg/kg β-NMN-pretreated mice (cf. Figure 3a).

As shown in Fig.5b, there was significant dose-related variation in the total calcium concentration increases in the CTX ($F = 7.92$, $p < 0.005$). However, there were no significant effects on CA1 regions ($F = 2.54$). There was also a significant dose-dependent variation in the response to the second and third 80K challenges in CTX ($F = 8.67$, $p < 0.005$ and $F = 5.73$, $p < 0.01$, respectively). However, we can't find significant effect on CA1 region (see F value in Fig.5 c right panel).

As shown in Fig.5d, there were no significant effects on the recovery within 5 min washing.

3.5 Effects of TND1128 on $[Ca^{2+}]_{mit}$ changes induced by the administration of 80K

Fig.6a shows the $[Ca^{2+}]_{mit}$ dynamics induced by three times 80K stimulation on X-Rhod-1 loaded control mice (n = 8) and on TND1128 (0.01, 0.1 or 1.0 mg/kg; n = 5 each) pretreated mice. The responses of the $[Ca^{2+}]_{mit}$ induced by 80K stimulation in control and each three dose of TND1128 treated mice showed dose-dependent mitigation. As shown in Fig.6b, there was a significant dose-response variation in the total $[Ca^{2+}]_{mit}$ changes (Fig. 6 b: CTX, $F = 5.29$, $p < 0.01$, CA1, $F = 7.98$, $p < 0.005$). We detected a statistically significant difference between the control $[Ca^{2+}]_{mit}$ response and that obtained from 1 mg/kg TND1128-treated mice in CTX and CA1 (Tukey test, $p < 0.01$). There were much evident dose-dependent mitigating effects in the CA1 region (Tukey test, $P < 0.005$, between control and 1.0 mg/kg treated mice). TND1128 resulted in a dose-dependent decrease in each set from the first to the third stimulation by 80K in CTX and CA1 (confer F and p values in Fig.6 c; asterisk indicates a statistically significant difference between the doses detected by the Tukey test).

There was no significant difference in the recovery rate (see F values shown in Fig.5 d).

4. Discussion

4.1 Experimental methods for evaluating the effects of compounds on energy production mechanisms

Screening for drugs that promote energy production in the brain requires creating highly energy-demanding conditions. We developed this condition in mouse brain slice preparation with the repeated administration of 80K ACSF, which severely activates the voltage-operated Ca^{2+} channel (VOCC). Since neurons express a high density of VOCC on their plasma membrane, the severe Ca^{2+} increase observed in the slice preparation may reflect the situation of neuronal cells requiring energy. However, the intracellular Ca^{2+}-overload induced by this method is not so severe as to cause a

mitochondrial permeability transition (MPT) that is known to be involved in cell apoptosis.[27]

As shown in Fig.7, many critical energy-dependent types of molecules to keep the homeostasis of $[Ca^{2+}]_{cyt}$. Among them, the most critical one is the Ca^{2+} pump (ATPase) distributed on the plasma membrane, and on the endoplasmic reticulum (ER).[28] The energy-dependent Na^+/K^+ active transporter (Na^+/K^+ Pump) also participates in this recovery process by reducing the given depolarization.[29,30]

The $[Ca^{2+}]_{cyt}$ dynamics in control preparations looked as though they exceeded the Ca^{2+} concentration measurement limit of Fura-4F as a Ca^{2+} indicator. However, the Kd of Fura-4F is 770 nM, which can detect $[Ca^{2+}]_{cyt}$ at concentrations of more than 10 μM.[31] Therefore, the feature of the $[Ca^{2+}]_{cyt}$ dynamics observed here may reflect the limit of Ca^{2+} pump (ATPase) in Ca^{2+} extrusion because of energy deficiency. On the other hand, we could detect an obvious increase in $[Ca^{2+}]_{mit}$ during 80K stimulation using X-Rhod-1 with Kd$[Ca^{2+}]$(700 nM), which reflects the participation of the mitochondrial Ca^{2+} uniporter（MCU）activated by extreme elevation of $[Ca^{2+}]_{cyt}$ (Fig.7). [32]

The dynamic features of $[Ca^{2+}]_{cyt}$ and $[Ca^{2+}]_{mit}$ in neurons induced by severe depolarization are appropriate indicators to estimate the energy dynamics occurring in neuronal cells. The delay on the increase in $[Ca^{2+}]_{cyt}$ observed in the first 80K may be due to cell swelling induced by the massive influx of Na^+, together with water by the activation of voltage-operated Na^+ channel (VONC).[33,34] We can detect the cell swelling as a transient decrease in Fura-4F fluorescence induced by 360 nm excitation (F360), which is insensitive to the concentration of Ca^{2+}.[21] We found TND1128 could reduce the swelling (Supplemental Data Fig.2) .

4.2 Effects of β-NMN on the increase of $[Ca^{2+}]_{cyt}$ and $[Ca^{2+}]_{mit}$ induced by 80K ACSF

As shown in Fig.3a, the total $[Ca^{2+}]_{cyt}$ increase and the increase induced by the second and third 80K in CTX, but not in CA1 significantly mitigated by the highest dose of β-NMN. (Fig.3 b and c). However, we could not obtain significant effects of β-NMN on $[Ca^{2+}]_{mit}$ dynamics in CTX and CA1 (Fig.4 b, c). We will be able to find more evident effects of β-NMN by using much higher doses, such as 500 mg/kg, as already reported.[11,13] However, we believe that the results obtained at the dose used in the present study are sufficient to serve the intended purpose of knowing that β-NMN is a precursor of NAD^+, and the recovery of $[Ca^{2+}]_{cyt}$ may be due to the intervention of this compound in the ATP production process inside cells.

We showed the recovery index which obtained as a ratio between the level of $[Ca^{2+}]_i$ during 80K stimulation and that obtained during washing out. Although we

expected it as a good indicator for the evaluation of drug effects on $[Ca^{2+}]_{cyt}$ extrusion, this values obtained from the second and the third 80K stimulation are stable in the cytoplasm and mitochondria. This value seems to show the stability of cellular Ca^{2+} delivery, which is promoted by mitochondrial Ca^{2+} regulation depending on MCU and the anti-porter (NCLX) (Fig.7).[35,36]

4.3 Effects of TND1128 on the elevation of $[Ca^{2+}]_{cyt}$ and $[Ca^{2+}]_{mit}$ induced by 80K ACSF

We found that TND1128 significantly reduced cytoplasmic and mitochondrial calcium elevation due to 80K stimulation (Fig. 5 and Fig. 6). The results confirmed that TND1128 contributes to cellular energy metabolism as a cofactor for the energy production process in the brain. Since this compound is highly hydrophobic, it would pass through the blood-brain barrier and penetrate neuronal cells.[37]

The effects of TND1128 on mitochondrial responses to 80K stimulation were evident in both CTX and CA1. The results mean that a large amount of Ca^{2+} flowing into neurons via the activated VOCC during 80K stimulation is immediately pumped out by energy-dependent Ca^{2+}-Pumps (ATPase) distributed on the plasma membrane and endoplasmic reticulum (Fig.7). Powerful energy supply due to the effect of TND1128, neurons could easily avoid the intense crisis of 80K stimulation. The mechanisms of action of TND1128 seemed to be similar to those of β-NMN. Of note is the effective concentration of less than 1/100 of β-NMN. This low effective concentration is due to the hydrophobicity and chemical stability of TND1128.

4.4 The mechanism of action of TND1128 and its possible efficacy as a drug for rescuing cells in low energy supply

Although NAD^+ has been attracting attention as an activator of *Sirtuin*,[8,38,39] this molecule is also critical as an essential coenzyme for providing phosphate to ADP in anaerobic and aerobic ATP production. Based on the present study, we would like to place TND1128 (5-DFla) as a critical molecule contributing to glycolysis systems in the cytoplasm and the electron transfer chain in mitochondria (Fig. 7).

These results indicate that an exogenous molecule can be incorporated into the electron transfer chain and participate in ATP synthesis. We guess the machinery involved in energy production in the cytoplasm and mitochondria is required considerable time for its incorporation into sites.

As mentioned in the introduction, the decline of the brain function with the neurodegenerative disorder is a type of ischemic neuronal cell deterioration due to blood

supply deficiency. We currently have no effective medicines for neurodegenerative diseases like AD, PD, or cerebral stroke. However, we can expect the probability of TND1128 as a drug that can reduce the shedding of neurons due to cerebrovascular disorders. It may be possible to suppress the progress of the pathological condition by physically impeding the neuron's activity by accumulating foreign bodies around neurons, like Aβ in the hippocampus and cerebral cortex in AD and α-synuclein in the dopaminergic neuron of the substantia nigra in PD. Furthermore, since Katsurabayashi et al. (2021) demonstrated the prominent branching of axons and dendrites in cultured neuronal cells by TND1128, we can expect the restoration of the lost neuronal network by this drug.[17]

We should add the other possibility of TND1128 as a hopeless brain neuronal disease drug. That is amyotrophic lateral sclerosis (ALS), a disease with a cardinal symptom of selective motor neuron loss by the excess superoxide because of the deficit of superoxide dismutase 1 (SOD1).[40] Currently, we have no effective medicine to rescue those patients. There may be a possibility of reducing the motor neuron loss due to the excess superoxide by TND1128 with an efficient reduction activity.

In addition to brain dysfunction, we can expect TND1128 to alleviate the crisis in patients with blood oxygen deficiency, such as pulmonary emphysema. Furthermore, this drug may have a critical possibility for emergency rescue of COVID-19, which causes many affected patients to die from respiratory disorders and its brain function-related sequelae.

Conclusion

The present study demonstrates that brain slices obtained from mice pretreated for 22 ± 2 hours with TND1128, a 5-deazaflavin derivative with a self-redox ability, can withstand severe conditions that require intense energy production. We recognized a similar effect in β-MMN used as the active control. These facts indicate that externally administered molecules with redox activity can be directly incorporated into anaerobic ATP production in the cytosol and aerobic condition through the electron transfer chain in mitochondria. TD1128 is highly hydrophobic and thus expected to transfer into the brain. We can confirm this expectation based on its effectiveness at a dose of less than 1 mg/kg (s.c.). The present results suggest the efficiency of TND1128 as a protective drug for a wide variety of cerebrovascular disorder-related diseases, including AD and PD, by enhancing the energy metabolism of individual neurons. Furthermore, TND1128 will rescue patients suffering from low oxygen uptake due to disorders of respiratory organs, such as COVID-19, and its brain function-related sequelae.

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Conflict of interest

This study was funded partially by Chemiteras Corporation, Kanagawa, Japan. However, the sponsor had no control over this work's interpretation, writing, or publication.

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Figure legends

Figure 1. Structures of βNMN and TND1128

a) β-NMN: a highly water-soluble compound with the phosphoribosyl-bond on the *N*-position of nicotinamide. This compound is proven to have an effective redox activity

b) TND1128: a hydrophobic compound synthesized as a 5-deazaflavin derivative, in which the *N* at the fifth position of the flavin structure was substituted by CH. This compound also involves nicotinamide structure in it. TND1128 bears efficient self-redox properties and works as an electron carrier in cells like NADH and $FADH_2$ as a reduced form of 5-DflaH_2.

Figure 2. Measurements of cytosolic and mitochondrial calcium in cells by two types of fluorescent calcium indicators responded to 80K ACSF in mouse slices

We obtained two-dimensional fluorescence images of the preparation by a ×4 objective lens (Plan N, Olympus) using a CCD camera (Orca-ER; Hamamatsu Photonics., Shizuoka, Japan) which processed by an image analyzer (AquaCosmos, ver. 2.6, Hamamatsu Photonics).

a) $[Ca^{2+}]_{cyt}$ was measured using mouse brain slice stained with Fura4F. Top row: F360/F380 time lapse images. Graph in the lower row: The reaction of F360/F380 was normalized to the value at the initial 80K stimulation (a fine black line shows the normalized base line). Red and blue solid lines show the time course of intracellular Ca^{2+} changes in the CTX and CA1 regions by F360/F380, respectively. The fine dotted line indicates the time course of the CTX and CA1 regions of F360, and the bold dotted line

indicates that of F380. 80K ACSF was applied for 5 minutes and was washed for 5 minutes. This procedure was repeated three times. The red arrow indicates the timing of the rise of $[Ca^{2+}]_{cyt}$ induced by the depolarization. The red asterisk indicates cell swelling.

b) Brain slice preparation obtained from the same mouse as (a) was stained using X-Rhod-1, a mitochondrial Ca^{2+} indicator. Top row: F580/F580 time lapse images. The 80K ACSF stimulation was repeated three times, similarly to (a), and normalized to the value at the time of the first 80K stimulus administration (a fine black line shows the normalized base line). The red line shows the response of the cerebral cortex, and the blue line shows that of the hippocampus.

Figure 3. The effects of 22 ± 2-hour spretreated β–NMN on the $[Ca^{2+}]_{cyt}$ dynamics induced by 80K ACSF in mouse brain preparations.

a) Brain slice preparations obtained from mice to which β-NMN was subcutaneously administered 22 ± 2 hours previously were stained with Fura4F and stimulated by 80K ACSF. From the top, Control (n = 8), βNMN 10 mg/kg (n = 5); β-NMN 30 mg/kg (n = 5) and β-NMN 100 mg/kg (n = 5). Solid line: cerebral cortex (CTX); Dotted line: hippocampal CA1 region. The mean time course of each group is shown with the SEM.

b) Dose-response relationship of β-NMN to a total calcium concentration increase for 30 minutes from the first 80K stimulus to after 5-min washing following the third stimulus (see Supplemental Figure 1 for the calculation method).

c) Dose-response relationships of β-NMN with the increase in $[Ca^{2+}]_{cyt}$ within 5 min of each three-time 80K stimulation.

d) Effects of β-NMN on recovery ratio obtained from the amount of $[Ca^{2+}]_{cyt}$ increase during each 80K administration to that obtained during 5 minutes of washing.
We expressed each data in each group as the dot plot with a mean diamond (green) which shows 95% confidence intervals, and a bar to show standard error (blue). We examined the statistical significance of each dataset by ANOVA and showed the F and p-value (statistically significant $p < 0.05$; n.s.: not significant). When we obtained a significant difference in the ANOVA, we analyzed the difference among a dataset by the Tukey method. When we found statistical significance between each data set, we put asterisks according to the significance rate as * $p < 0.05$. This statistical treatment applied the following Figures

Figure 4. The effects of 22 ± 2 hours pretreated β-NMN on the $[Ca^{2+}]_{mit}$ dynamics induced by 80K ACSF in mouse brain preparations.

a) Brain slice preparations from mice to which β-NMN was subcutaneously administered 22 ± 2 hours previously were stained with X-rhod-1, as a $[Ca^{2+}]_{mit}$ indicator, and stimulated by 80K ACSF in the same manner as shown in Fig3. We examined the effect of β-NMN on the dynamics of $[Ca^{2+}]_{mit}$. From the top, Control; n = 8), β-NMN 10 mg/kg (n = 5); β-NMN 30 mg/kg (n = 5) and β-NMN 100 mg/kg. (n = 5). Solid line: cerebral cortex (CTX); Dotted line: hippocampal CA1 region.

b) Dose-response relationship of β-NMN to a total $[Ca^{2+}]_{mit}$ increase for 30 minutes from the first 80K stimulus obtained from the data in (a) to washing for 5 minutes after the third stimulus.

c) Dose-response relationship of β–NMN to the increase in $[Ca^{2+}]_{mit}$ within 5 min of each three-time 80K stimulation.

d) Recovery ratio obtained from the amount of $[Ca^{2+}]_{mit}$ increase during each 80K administration to that obtained during 5 minutes of washing.

Figure 5. The effects of 22 ± 2-hours pretreated TND1128 on $[Ca^{2+}]_{cyt}$ dynamics induced by 80K ACSF in mouse brain preparations.

a) Brain slice preparations from mice to which TND1128 was subcutaneously administered 22 ± 2 hours previously were stained with Fura4F and stimulated by 80K-ACSF. From the top, Control (n = 8), TND1128 0.01 mg/kg (n = 5); TND1128 0.1 mg/kg (n = 5) and TND 1 mg/kg (n = 5). Solid line: cerebral cortex (CTX); Dotted line: hippocampal CA1 region.

b) Dose-response relationship of TND1128 to a total calcium concentration increase for 30 minutes from the first 80K stimulus to after 5 min washing the third stimulus.

c) Dose-response relationships of TND1128 with the increase in $[Ca^{2+}]_{cyt}$ within 5 min of each three-time 80K stimulation.

d) Recovery ratio obtained from the amount of $[Ca^{2+}]_{cyt}$ increase during each 80K administration to that obtained during 5 minutes of washing.

** and *** indicate statistical significance $p < 0.01$ and $p < 0.005$, respectively tested by Tukey method.

Figure 6. The effects of 22 ± 2-hours pretreated TND1128 on $[Ca^{2+}]_{mit}$ dynamics induced by 80K ACSF in mouse brain preparations.

a) Brain slice preparations from mice pretreated for 22 ± 2 hours were stained with X-Rhod-1, as a $[Ca^{2+}]_{mit}$ indicator, and stimulated by 80K ACSF in the same manner as shown in Fig3. We examined the effect of TND1128 on the dynamics of $[Ca^{2+}]_{mit}$. From

the top, Control (n = 8), TND1128 0.01 mg/kg (n = 5); TND1128 0.1 mg/kg (n = 5) and TND 1 mg/kg (n = 5). Solid line: cerebral cortex (CTX); Dotted line: hippocampal CA1 region. The mean time course of each group is shown with the SE.

b) Dose-response relationship of TND1128 to the total $[Ca^{2+}]_{mit}$ increase for 30 minutes from the first 80K stimulus obtained from the data in (a) to washing for 5 minutes after the third stimulus.

c) Dose-response relationships of TND1128 to the increase in $[Ca^{2+}]_{mit}$ within 5 min of each three-time 80K stimulation.

d) Recovery ratio obtained from the amount of $[Ca^{2+}]_{mit}$ during each 80K administration to that obtained during 5 minutes of washing.

*, ** and *** indicate statistical significance $P<0.05$, $P< 0.01$ and $P<0.005$, respectively tested by Tukey method.

Figure 7 Schematic illustration of the functional molecules involved in Ca^{2+} dynamics in the neuron.

The area shown by rea shading shows the distribution of functional molecules participating in the inflow of Ca^{2+} into the cytoplasm and mitochondria induced by depolarization due to 80K stimulation. The blue shading area shows the existence of energy-dependent types of machinery (Ca^{2+} pumps on the plasma membrane and endoplasmic reticulum; Na^+/K^+ pump on the plasma membrane) participating in the recovery of Ca^{2+} concentrations during washing by normal ACSF. Green arrows show the possible sites of TND1128 and β-NMN. The effects of TND1128 on the mitigating $[Ca^{2+}]_{cyt}$ and $[Ca^{2+}]_{mit}$ are far more extensive than those of β-NMN.

VOCC, voltage-operated Ca^{2+} channel; VONC, voltage operated Na^+ channel, MCU: mitochondrial Ca^{2+} uniporter; NCLX, $Na^+/Ca^{2+}/Li^+$ exchanger; NCX, Na^+/Ca^{2+} exchanger; ETC, electron transfer chain; ATPS, ATP synthase; ER; endoplasmic reticulum.