

# NMDA-receptor-initiated modulatory effect on GABA transporter current: experiment and modelling

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**Abstract**—Whole cell recording was applied to investigate the modulatory effect of N-methyl-D-aspartate (NMDA) on  $\gamma$ -aminobutyric acid (GABA) transporter current on enzymatically isolated horizontal cells of carp retina, and a model containing endoplasmic reticulum (ER) membrane process and plasma membrane process was constructed for quantitative analyses of the modulatory effect of NMDA and changes of intracellular  $\text{Ca}^{2+}$ . The GABA transporter current elicited by GABA (1 mM) was decreased by pre-application of NMDA (0.1 mM). The suppressive effect could be attenuated when the intracellular  $\text{Ca}^{2+}$  store was depleted after the cell had been pre-incubated with 10  $\mu\text{M}$  thapsigargin, a selective blocker of ER  $\text{Ca}^{2+}$ -ATPase. The model analyses suggest that application of NMDA caused a transient release of  $\text{Ca}^{2+}$  from  $\text{Ca}^{2+}$  store, which was important for the NMDA modulation of GABA transporter.

**Keywords**—NMDA receptor; calcium store; GABA transporter; horizontal cell

## I. INTRODUCTION

In the vertebrate retina, luminosity-type horizontal cells are the interneurons that use  $\gamma$ -aminobutyric acid (GABA) as the neurotransmitter. GABA transporter on the horizontal cell is responsible for GABA uptake and release in reverse modes [1]. The horizontal cells receive direct input from photoreceptors, which are believed to use glutamate as their neurotransmitter [2]. It is generally accepted that the glutamate receptors on horizontal cell are  $\alpha$ -amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptors. Recently, it has been found that another glutamate receptor subtype, N-methyl-D-aspartate (NMDA) receptor, is expressed in the carp retinal horizontal cell [3]. NMDA receptors are known to be involved in a variety of physiological and pathological processes in the central nervous system. NMDA receptors are more permeable to  $\text{Ca}^{2+}$  than non-NMDA receptors, and produce long-lasting effects on the neurons when activated. Regulation of cytoplasmic free  $\text{Ca}^{2+}$  concentration is important for the NMDA modulation.

In the present work, the modulatory effect that the NMDA conductance exerts on GABA transporter-mediated current was investigated in freshly dissociated horizontal cells of carp retina using patch clamp technique. To investigate the possible mechanism of such inhibition, and the possible relationship among NMDA receptor, GABA transporter, intracellular free  $\text{Ca}^{2+}$ , and endoplasmic  $\text{Ca}^{2+}$  store in the horizontal cell, a

mathematical model was constructed, and the model output was compared with the experimental results.

## II. MATERIALS AND METHODS

### A. Cell isolation

Horizontal cells were enzymatically dissociated from retinas of adult carp (*Carassius Auratus*, 15-20 cm body length), as described previously [4]. Briefly, retinas were isolated from carp, and incubated for 20 min at room temperature in 4 ml Hank's solution with 25 U/ml papain and 4 mg L-cysteine. The retinas were rinsed and stored in the Hank's solution at 4 °C. Cells were freshly dissociated from the retina pieces by gentle trituration in Ringer's solution. H1 horizontal cell was identified by its characteristic morphology.

### B. Whole cell recording and drug application

Whole cell recordings, voltage-clamped at -60 mV, were obtained using 5-8 M $\Omega$  patch pipettes pulled from borosilicate glass (Sutter Instrument Inc., USA) using a horizontal puller (P87, Sutter Instrument Inc., USA). The pipette was filled with intracellular solution, mounted on a motor-driven micromanipulator (MC1000e, SD Instrument Inc., USA), and was connected to a patch amplifier (Axopatch 200B, Axon Instrument Inc., USA). An agar/NaCl bridge connected to the recording chamber with an Ag/AgCl wire inside was used as a reference electrode. Fast capacitance, cell capacitance transients, and 70% of the series resistance of the recording electrode were compensated. Data acquisition was performed using AxoScope software (Axon Instrument Inc., USA), with sample rate being 1 kHz and was lowpass filtered (0 ~ 1 kHz). The recorded data were analyzed by Clampfit 9.2 software (Axon Instrument Inc., USA).

### C. Solutions

Hank's solution contained (in mM) 120.0 NaCl, 3.0 KCl, 0.5  $\text{CaCl}_2$ , 1.0  $\text{MgSO}_4$ , 1.0 Na-pyruvate, 1.0  $\text{NaH}_2\text{PO}_4$ , 0.5  $\text{NaHCO}_3$ , 20.0 HEPES and 16.0 glucose.  $\text{Mg}^{2+}$ -free Ringer's solution contained (in mM) 120.0 NaCl, 5.0 KCl, 2.0  $\text{CaCl}_2$ , 10.0 HEPES and 16.0 glucose. Thapsigargin was prepared in DMSO and diluted to its final concentration in Ringer's solution (DMSO < 0.5%). PTX, NMDA and GABA were dissolved in Ringer's solution. The pH value for the perfusate

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was adjusted to 7.4 with NaOH. The intracellular solution for patch electrode contained (in mM) 140.0 CsCl, 0.05 CaCl<sub>2</sub>, 1.0 MgSO<sub>4</sub>, 0.5 EGTA, 10.0 HEPES. The pH value was adjusted to 7.3 with CsOH. All the drugs were purchased from Sigma (St. Louis, MO, USA).

#### D. Modeling

A spatially uniform model is constructed to describe the relationship between NMDA receptor, GABA transporter and [Ca<sup>2+</sup>]<sub>i</sub>. The model contains three compartments, i.e., extracellular environment, cytosol and endoplasmic reticulum (ER). To simulate the experimental condition, the membrane potential is fixed at -60 mV. The Ca<sup>2+</sup> influx across the plasma membrane is only via NMDA receptor. The Ca<sup>2+</sup> efflux across the plasma membrane is due to Ca<sup>2+</sup> pump and Na<sup>+</sup>/Ca<sup>2+</sup> exchanger. The ryanodine receptor (RyR) in the endoplasmic reticulum (ER) is responsible for the Ca<sup>2+</sup>-release from ER. The sarco-endoplasmic reticulum Ca<sup>2+</sup>-ATPase determines the Ca<sup>2+</sup>-refill into ER. Also, there is passive leak of Ca<sup>2+</sup> from ER.

The changes of calcium in the cytosol can be described as:

$$\frac{d[Ca^{2+}]_i}{dt} = f_{cyt} \cdot (\phi_{in} - \phi_{out} + \phi_{ER}) \quad (1)$$

where  $f_{cyt}$  is the Ca<sup>2+</sup> buffering coefficient of cytoplasm by Ca<sup>2+</sup>-binding proteins.  $\phi_{in}$  and  $\phi_{out}$  are the Ca<sup>2+</sup> that influx and efflux across the plasma membrane respectively.  $\phi_{ER}$  is the Ca<sup>2+</sup> released from ER. They can be described as:

$$\phi_{in} = \frac{I_{Ca^{2+},NMDA}}{2FV_{cyt}} \quad \phi_{out} = \frac{I_{pump} + I_{Ca^{2+},exchanger}}{2FV_{cyt}} \quad (2)$$

$$\phi_{ER} = C_{ER,rel} - C_{ER,fil} + C_{ER,leak}$$

where  $V_{cyt}$  is the volume of the cell;  $F$  is the Faraday constant;  $C_{ER,rel}$  is the flow rate of Ca<sup>2+</sup> release from the ER into the cytosol;  $C_{ER,fil}$  is the rate of Ca<sup>2+</sup>-refilling into the ER Ca<sup>2+</sup> pool;  $C_{ER,leak}$  is the passive leak of Ca<sup>2+</sup> from the ER into the cytosol [5].

$C_{ER,rel}$ ,  $C_{ER,fil}$  and  $C_{ER,leak}$  are further described as:

$$C_{ER,rel} = v_{rel}(P_{O_1} + P_{O_2})([Ca^{2+}]_{ER} - [Ca^{2+}]_i)$$

$$C_{ER,fil} = -v_{fil} \frac{[Ca^{2+}]_i^2}{[Ca^{2+}]_i^2 + k_{fil}^2}$$

$$C_{ER,leak} = v_{leak}([Ca^{2+}]_{ER} - [Ca^{2+}]_i) \quad (3)$$

Gating properties of RyR on intracellular calcium store can be described by a four-state kinetic model [6]:

$$\frac{dP_{C_1}}{dt} = -K_a^+[Ca^{2+}]_i^n + K_a^- P_{O_1}$$

$$\frac{dP_{O_2}}{dt} = K_b^+[Ca^{2+}]_i^m P_{O_1} - K_b^- P_{O_2}$$

$$\frac{dP_{C_2}}{dt} = K_c^+ P_{O_1} - K_c^- P_{C_2}$$

$$P_{O_1} + P_{O_2} + P_{C_1} + P_{C_2} = 1 \quad (4)$$

where  $P_{C_1}$  and  $P_{C_2}$  are RyRs at closed states, whereas  $P_{O_1}$  and  $P_{O_2}$  are RyR at open states;  $K_a^+$ ,  $K_a^-$ ,  $K_b^+$  and  $K_b^-$  are relevant rate constants.

Ca<sup>2+</sup> flux across the internal Ca<sup>2+</sup> store (ER) can be described as:

$$\frac{d[Ca^{2+}]_{ER}}{dt} = -f_{er} \cdot c \cdot (ER_{rel} - ER_{fil} + ER_{leak}) \quad (5)$$

where  $[Ca^{2+}]_{ER}$  is the Ca<sup>2+</sup> concentration in ER;  $f_{er}$  is the Ca<sup>2+</sup> buffering coefficient of ER;  $c$  is the ratio of the cell volume (excluding ER) and the ER volume.

The activities of Ca<sup>2+</sup> pump and Na<sup>+</sup>/Ca<sup>2+</sup> exchanger are major source of Ca<sup>2+</sup> efflux across plasma membrane. The Ca<sup>2+</sup> currents induced by these processes can be described as [7]:

$$I_{pump} = \frac{2F \cdot V_{cyt}}{S_{cyt}} \cdot \frac{A_{pump}[Ca^{2+}]_i}{K_{pump} + [Ca^{2+}]_i} \quad (6)$$

$$I_{Ca^{2+},exchanger} = -2K([Na^+]_i^3[Ca^{2+}]_o \exp((n-2) \cdot r \cdot V_m \cdot 10^{-3} \frac{F}{RT}) - [Na^+]_o^3[Ca^{2+}]_i \exp(-(n-2) \cdot (1-r) \cdot V_m \cdot 10^{-3} \frac{F}{RT}))$$

where  $[Na^+]_i$  and  $[Na^+]_o$  are intracellular and extracellular sodium concentration, whereas  $[Ca^{2+}]_i$  and  $[Ca^{2+}]_o$  are intracellular and extracellular calcium concentration, respectively;  $F$ ,  $T$  and  $R$  are the Faraday constant, the absolute temperature and the gas constant, respectively.

The Ca<sup>2+</sup> current induced by NMDA can be described as [8]:

$$\frac{dR_o}{dt} = \alpha C_{NMDA}(t)R_c - \beta R_o$$

$$R_c + R_o = 1 \quad (7)$$

$$E_{Ca^{2+}} = \frac{RT}{2F} \ln \left( \frac{[Ca^{2+}]_o}{[Ca^{2+}]_i} \right)$$

$$I_{Ca^{2+},NMDA} = g_{max} \cdot fca(V_m - E_{Ca^{2+}})$$

where  $C_{NMDA}$  is the concentration of NMDA;  $E_{Ca^{2+}}$  is the equilibrium potential of Ca<sup>2+</sup>.

The GABA-transporter-mediated current can be described as [9]:

$$\frac{dR_m}{dt} = -\alpha_m \cdot [Ca^{2+}]_i^z \cdot R_m + \beta_m \cdot R_n$$

$$R_m + R_n = 1 \quad (8)$$

$$I_{trans} = v \cdot R_m \cdot \frac{I_{max}}{1 + \left( \frac{EC_{50}}{C_{GABA}(t)} \right)^n}$$

where  $R_m$  and  $R_n$  are the activation and the inactivation variables, respectively;  $I_{max}$  is the maximum current mediated by GABA transporter;  $EC_{50}$  is median effective concentration of GABA, and Hill coefficient  $n$  is set as 0.95.

### III. RESULTS

#### A. NMDA modulatory effect on GABA transporter current

Whole cell recordings were performed on isolated retinal H1 horizontal cell, which was voltage clamped at -60 mV. The GABA-elicited current was mediated by GABA transporter in the presence of 300 μM picrotoxin (PTX), a potent antagonist of GABA<sub>A</sub> and GABA<sub>C</sub> receptors. In the example presented in Fig. 1 (a), application of 1 mM GABA in the presence of PTX elicited an inward current of 182.1 pA, which was taken as a control. Then 100 μM NMDA was superfused for 50 sec. After NMDA was washed out by the Ringer's solution for 5 sec, GABA (1 mM) was applied again. The pre-application of

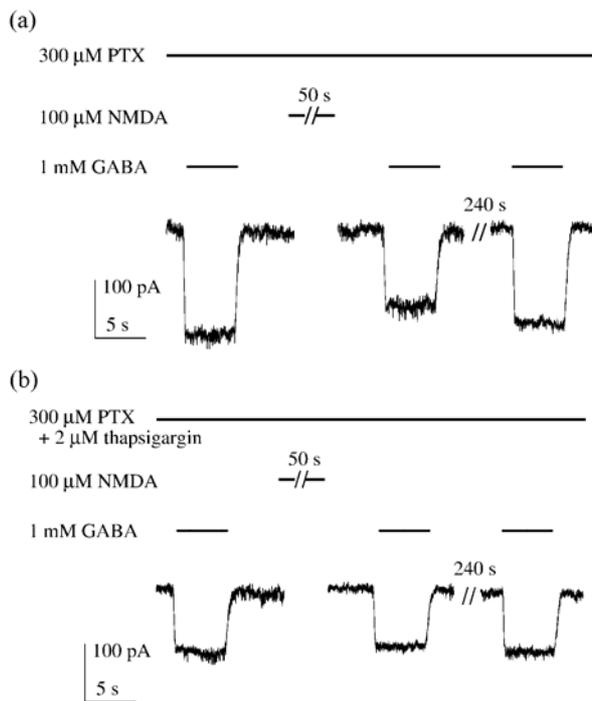


Figure 1. NMDA modulatory effect on GABA transporter current

NMDA decreased the GABA-elicited current to 136.8 pA. After superfusion with the PTX-containing Ringer's solution for another 4 min, the GABA-elicited current was mostly recovered (174.6 pA).

The activation of NMDA receptor can cause  $\text{Ca}^{2+}$ -induced- $\text{Ca}^{2+}$ -release (CICR) from endoplasmic reticulum. To further examine the contribution of CICR in the NMDA receptor-initiated GABA transporter inhibition, thapsigargin (2  $\mu\text{M}$ ), a selective blocker of endoplasmic reticulum  $\text{Ca}^{2+}$ -ATPase [9-10], was presented in the bath solution 3 min prior to the experimental recordings. Fig. 1 (b) gives an example from a cell, in which the GABA transporter-mediated currents were 111.0 pA, 100.6 pA, and 106.1 pA (control, after NMDA application, and recovery, respectively). The reduction of GABA-elicited currents after pre-application of NMDA was significantly attenuated as compared to that measured in the normal Ringer's solution as shown in Fig.1 (a).

#### B. Simulation of NMDA modulatory effect on GABA transporter current

Using the parameter values chosen referring to previous findings [5-9], the changes of the GABA transporter current were calculated by applying the model. Fig. 2 (a) presents the GABA transporter-mediated currents measured during control, after the pre-application of NMDA and recovery (135.1 pA, 105.6 pA, and 135.1 pA). Pre-application of NMDA decreases the GABA transporter current to 78.2% of the control level.

In order to examine the contribution of  $\text{Ca}^{2+}$  release from intracellular  $\text{Ca}^{2+}$  store,  $v_{\text{fil}}$  in equation (3) is set to be zero to

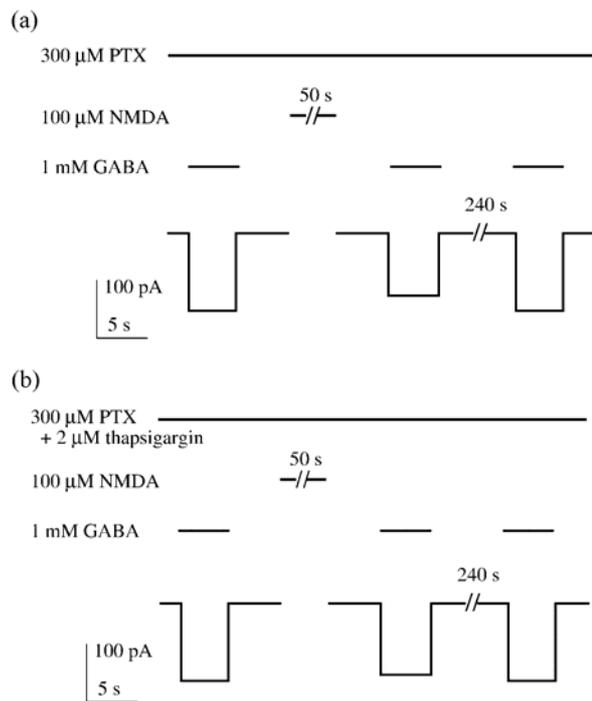


Figure 2. Simulation of NMDA modulatory effect on GABA transporter current using the computational model of horizontal cell.

simulate the inhibitory effect of thapsigargin exerts on the endoplasmic reticulum  $\text{Ca}^{2+}$ -ATPase. Due to the passive leak of  $\text{Ca}^{2+}$  from the ER into the cytosol, the intracellular  $\text{Ca}^{2+}$  store is depleted. Fig. 2 (b) shows the GABA transporter-mediated currents during control, after the pre-application of NMDA and recovery (135.1 pA, 121.5 pA, and 135.1 pA) after the intracellular  $\text{Ca}^{2+}$  store is eliminated in the computational model. The pre-application of NMDA decreases the GABA transporter current to 90.0% of the control level. The modulatory effect of NMDA is attenuated by depleting the  $\text{Ca}^{2+}$  store.

Fig. 3 presents the dynamic changes of  $[\text{Ca}^{2+}]_i$  in the model. The solid line shows the change of intracellular free  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_i$ ) such that the application of NMDA (100  $\mu\text{M}$ ) resulted in an increase of  $[\text{Ca}^{2+}]_i$ , with the transient peak reaching 3.5  $\mu\text{M}$ , and the following sustained plateau at 1.7  $\mu\text{M}$ . The peak of  $[\text{Ca}^{2+}]_i$  is elicited by  $\text{Ca}^{2+}$ -induced- $\text{Ca}^{2+}$ -release from intracellular  $\text{Ca}^{2+}$  store, since it can be eliminated when intracellular  $\text{Ca}^{2+}$  store is depleted. The dotted line in Fig. 3 shows the change of  $[\text{Ca}^{2+}]_i$  in the presence of thapsigargin. In this case, the application of NMDA causes an increase of  $[\text{Ca}^{2+}]_i$  with its final steady level of 1.7  $\mu\text{M}$ . The peaky increase in  $\text{Ca}^{2+}$  is eliminated. Although a steady state  $[\text{Ca}^{2+}]_i$  elevation can be reached either during control and during  $\text{Ca}^{2+}$  store depletion, the NMDA modulatory effect on GABA transporter current are different. It may imply that the transient increase of  $[\text{Ca}^{2+}]_i$  is important for the NMDA modulatory effect.

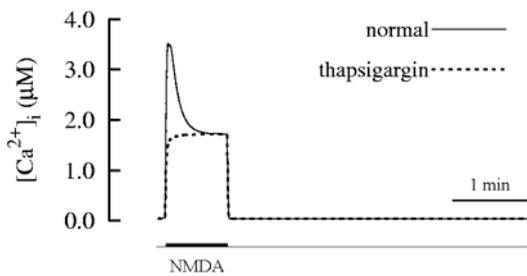


Figure 3. Simulation of the changes of  $[Ca^{2+}]_i$  initiated by application of NMDA

#### IV. DISCUSSION

In the present study, the experimental results observed using patch clamp technique demonstrate that the application of exogenous NMDA can down-regulate the GABA transporter-mediated current in freshly isolated H1 cells of carp retina. The NMDA modulatory effect depends upon the presence of  $Ca^{2+}$ , since the inhibitory effect was significantly attenuated when the intracellular  $Ca^{2+}$  store was depleted. The computational results demonstrate that the application of NMDA results in an increase of  $[Ca^{2+}]_i$  with a transient peak and a following sustained plateau. It also suggests that the transient peak, which is caused by the  $Ca^{2+}$ -release from the intracellular  $Ca^{2+}$  store, is more important than the following sustained plateau for the NMDA modulatory effect on the GABA transporter.

In previous studies, it was reported that glutamate regulates GABA uptake in horizontal cell of skate retina through a  $Ca^{2+}$ -dependent process.  $Ca^{2+}$ -influx via AMPA receptor down-regulates the GABA uptake of the transporter [12]. In addition to AMPA receptors, it was recently found that functional NMDA receptor is expressed in carp H1 type horizontal cells [3]. In the present study, application of NMDA inhibited the GABA transporter-mediated current in freshly dissociated horizontal cells of carp retina. And it is well known that NMDA receptors are more permeable to  $Ca^{2+}$  than AMPA receptors. So it is necessary to investigate the role of intracellular  $Ca^{2+}$  in the NMDA modulatory effect on GABA transporter.

Under our experimental condition, activation of glutamate receptors in retinal horizontal cells elicited an increase of  $[Ca^{2+}]_i$ , which may be due to  $Ca^{2+}$  entry through the NMDA channel, and the following  $Ca^{2+}$ -induced- $Ca^{2+}$ -release from intracellular stores. In our previous studies using calcium imaging technique, it was observed that application of AMPA [5] or NMDA [11] resulted in an increase of  $[Ca^{2+}]_i$  with a transient peak and a following sustained plateau in isolated

retinal horizontal cells. The experimental and computational results demonstrated that  $Ca^{2+}$  store is significantly involved in the neuronal  $Ca^{2+}$  dynamics, and  $Ca^{2+}$ -induced- $Ca^{2+}$ -release from intracellular  $Ca^{2+}$  store should be responsible for the initial transient elevation of  $[Ca^{2+}]_i$ . In the present work, the model analyses results suggest that during application of NMDA, the initial transient increase of  $[Ca^{2+}]_i$  is more important than the subsequent sustained plateau, although the sustained phase is longer. It implies that the high concentration of  $[Ca^{2+}]_i$  during the initial transient increase of  $[Ca^{2+}]_i$  contributes more to the inhibition of GABA transporter and produces long lasting effects. The increased intracellular  $Ca^{2+}$  store-derived  $[Ca^{2+}]_i$  signaling allows the horizontal cell to amplify its modulatory effect on GABA transporter.

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