

## Intracellular Ca<sup>2+</sup> regulates spike encoding at cortical GABAergic neurons and cerebellar Purkinje cells differently

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### ABSTRACT

Spike encoding at GABAergic neurons plays an important role in maintaining the homeostasis of brain functions for well-organized behaviors. The rise of intracellular Ca<sup>2+</sup> in GABAergic neurons causes synaptic plasticity. It is not clear how intracellular Ca<sup>2+</sup> influences their spike encoding. We have investigated this issue at GFP-labeled GABAergic cortical neurons and cerebellar Purkinje cells by whole-cell recording in mouse brain slices. Our results show that an elevation of intracellular Ca<sup>2+</sup> by infusing adenophostin-A lowers spike encoding at GABAergic cortical neurons and enhances encoding ability at cerebellar Purkinje cells. These differential effects of cytoplasmic Ca<sup>2+</sup> on spike encoding are mechanistically associated with Ca<sup>2+</sup>-induced changes in the refractory periods and threshold potentials of sequential spikes, as well as with various expression ratios of CaM-KII to calcineurin in GABAergic cortical neurons and cerebellar Purkinje cells.

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### Introduction

Sequential action potentials at the neurons are one type of essential neural codes, and the different spike patterns encode various messages to control the well-organized behaviors and cognition [1–6]. It is not clear whether neuronal spike patterns undergo the plasticity under the physiological conditions, similar to the plasticity of synaptic transmission that underlies learning and memory [1,7–12]. If it is a case, do the mechanisms underlying synaptic plasticity, such as intracellular Ca<sup>2+</sup> level [1,6,13,14], lead to the plasticity of spike patterns?

GABAergic inhibitory interneurons, despite a small population in the brain, play an important role in maintaining the functional homeostasis of the brain [15–19]. There are two kinds of morphologically different GABAergic neurons in the brain, interneurons in cerebral cortex and Purkinje cells in cerebellum. The former displays enriched axonal arbors and less dendrite, whereas the latter shows enriched dendrital branches and a single axon [20,21]. Additionally, spike patterns in these two kinds of neurons are different [15,20,21]. Do the signaling pathways regulate spike encoding differently? We investigated these questions in cortical GABAergic neurons, which are genetically labeled with GFP, and

cerebellar Purkinje neurons by whole-cell recordings and immunocytochemistry in mouse brain slices.

### Methods and materials

**Brain slices and neurons.** Cortical and cerebellar slices (400 μm) were made from FVB-Tg(Gad GFP)4570Sw/J mice (Jackson Lab., Bar Harbor, ME 04609, USA) in postnatal day 17–22. Mice were anesthetized by inhaling isoflurane and decapitated with a guillotine. Cortical slices were cut with a Vibratome in oxygenated (95% O<sub>2</sub> and 5% CO<sub>2</sub>) artificial cerebrospinal fluid (ACSF) in the concentrations (mM) of 124 NaCl, 3 KCl, 1.2 NaH<sub>2</sub>PO<sub>4</sub>, 26 NaHCO<sub>3</sub>, 0.5 CaCl<sub>2</sub>, 4 MgSO<sub>4</sub>, 10 dextrose, and 5 HEPES, pH 7.35 at 4 °C. The slices were held in (95% O<sub>2</sub> and 5% CO<sub>2</sub>) ACSF (124 NaCl, 3 KCl, 1.2 NaH<sub>2</sub>PO<sub>4</sub>, 26 NaHCO<sub>3</sub>, 2.4 CaCl<sub>2</sub>, 1.3 MgSO<sub>4</sub>, 10 dextrose, and 5 HEPES, pH 7.35) at 25 °C for 2 h. A slice was then transferred to a submersion chamber (Warner RC-26G) that was perfused with ACSF oxygenated at 31 °C for whole-cell recording [17,18,22–24]. Chemical reagents were from Sigma. The entire procedures were approved by IACUC in Anhui, China.

The cortical GABAergic neurons for whole-cell recording in layer II and III of sensory cortex were selected based on GFP-labeled cells under fluorescent microscope (Nikon, FN-E600), in which an excitation wavelength was 488 nm. GABAergic Purkinje cells in cerebellum were selected based on their morphology under DIC microscope (Nikon, FN-E600). These neurons demonstrated

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the typical properties of interneurons, such as fast-spiking and less adaptation in spike amplitude and frequency [15,17,18,20,21,23,24].

**Whole-cell recording.** Electrical signals were recorded by using an Axoclamp-2B amplifier under current-clamp, and were inputted into pClamp 9 (Axon Instrument Inc., Foster CA, USA) for data acquisition and analysis. The output bandwidth in the amplifier was 3 kHz. Spike patterns were evoked by depolarization current pulses, in which the amplitude and duration were based on the aim of experiments. Pipettes for whole-cell recordings were filled with the standard solution that contained (mM) 150 K-gluconate, 5 NaCl, 5 HEPES, 0.4 EGTA, 4Mg-ATP, 0.5 Tris-GTP, and 5 phosphocreatine (pH 7.35 adjusted by 2 M KOH). Fresh pipette solution was filtered with centrifuge filters (0.1  $\mu$ m) before the use, and osmolarity was 295–305 mOsmol. Pipette resistance was 5–6 M $\Omega$ .

The intrinsic properties of GABAergic neurons in our studies include the threshold potentials (V<sub>t</sub>) of firing spikes and absolute refractory periods (ARP) following each spike. V<sub>t</sub>s are a start voltage of spike rising phase [15,22,25]. The ARP of sequential spikes are measured by injecting multiple depolarization-current pulses (3 ms) into GABAergic neurons after each of spikes (Fig. 2). By changing inter-pulse intervals, we define ARP as the duration from a complete spike to its subsequent spike at 50% probability [22]. Spike programming (capacity and timing precision) is represented as inter-spike interval (ISI) and standard deviation of spike timing (SDST), respectively.

Data were analyzed if the recorded neurons had the resting membrane potentials negatively more than –60 mV. The criteria for the acceptance of each experiment also included less than 5% changes in resting membrane potential, spike magnitude, and input resistance throughout each of experiments. Input resistance was monitored by measuring cell responses to the hyperpolarization pulses at the same values as the depolarization that evoked spikes. V<sub>t</sub>s, ARP and ISI are presented as mean  $\pm$  SE. The comparisons before and after applying adenophostin-A are done by *t*-test.

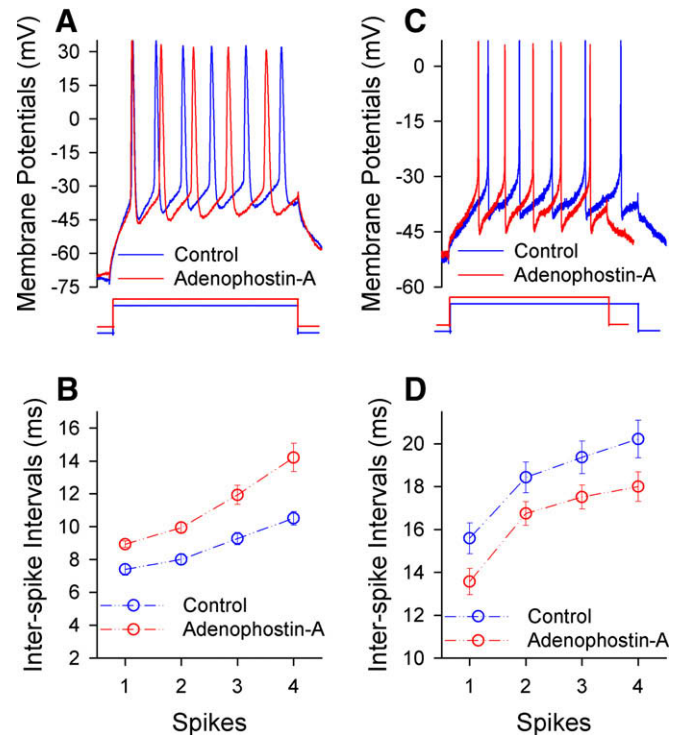
In the immunocytochemical study of calcineurin (CaN) and CaM-KII, FVB-Tg (GadGFP)4570Swn/J mice during postnatal days (PND) 20–24 were perfused with 4% paraformaldehyde in PBS through left ventricle and aorta until their body is hard. The brain was isolated, and further fixed in 4% paraformaldehyde PBS for 48 h. Brain tissue was sliced by a Vibratome at 40  $\mu$ m. The slices were incubated in monoclonal anti-CaN and polyclonal anti-CaMKII antibodies (Sigma, USA) at 4 °C for 48 h, and then were incubated in FTIC-coupled anti-mouse and red-fluorescent-coupled anti-rabbit antibodies (Sigma). The images of CaM-KII (red) and CaN (green) in cortical and cerebellar neurons were taken under confocal microscope (Olympus FV-1000, Japan), in which the parameters of laser beam and PMT are fixed for all experiments.

## Results

### Intracellular Ca<sup>2+</sup> regulates spike encoding at cortical and cerebellar GABAergic neurons differently

Whole-cell current-clamp recordings were conducted at GABAergic neurons in cortical slices and Purkinje cells in cerebellar slices. Cytoplasmic Ca<sup>2+</sup> was raised by infusing adenophostin-A, an agonist of IP3R, intracellularly via whole-cell pipettes [1]. Inter-spike intervals (ISI, an index of spike capacity) were measured by evoking action potentials with depolarization currents (100 ms).

Fig. 1A and B illustrates the effect of adenophostin-A on sequential spikes at cortical GABAergic neurons. The elevation of intracellular Ca<sup>2+</sup> appears to lower the number of spikes in a given time (Fig. 1A). The ISI values of spikes 1–2 to 4–5 are 7.47  $\pm$  0.35, 8.01  $\pm$  0.34, 9.28  $\pm$  0.37 and 10.51  $\pm$  0.41 ms under the control (blue symbols in Fig. 1B); and ISI's values are 8.9  $\pm$  0.2, 9.94  $\pm$  0.33,



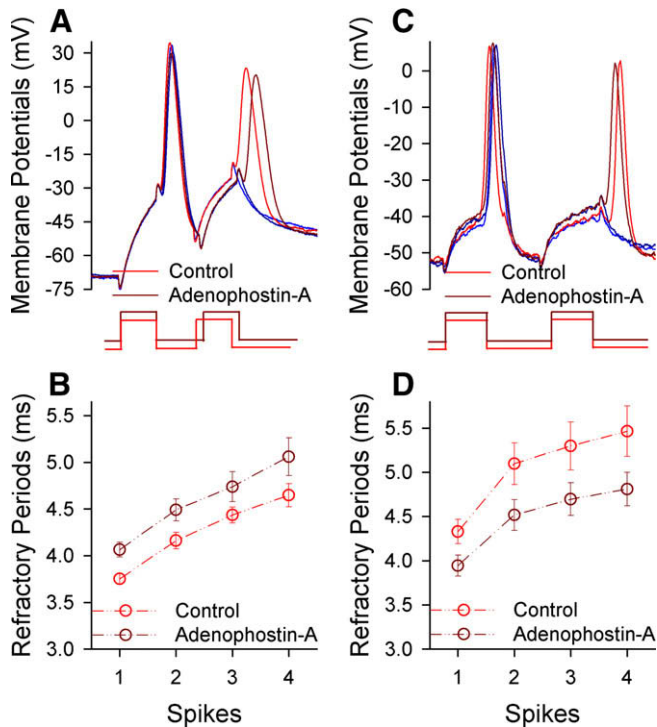
**Fig. 1.** Intracellular Ca<sup>2+</sup> attenuates spike encoding at cortical GABAergic neurons and enhances spike encoding at cerebellar Purkinje cells in brain slices from FVB-Tg(Gad GFP)4570Swn/J mice. (A) The superimposed waveforms of sequential spikes are evoked by depolarization pulses (100 ms) during infusing adenophostin-A (red trace) vs. control (blue trace) at cortical GABAergic neurons. (B) Inter-spike intervals (ISI) of sequential spikes under control (blue symbols) and adenophostin-A infusion (red symbols) at cortical GABAergic neurons. (C) The superimposed waveforms of sequential spikes are evoked by depolarization pulses during infusing adenophostin-A (red trace) vs. control (blue trace) at cerebellar Purkinje cells. (D) Inter-spike intervals (ISI) of sequential spikes under control (blue symbols) and adenophostin-A infusion (red symbols) at cerebellar Purkinje cells. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

11.94  $\pm$  0.57 and 14.21  $\pm$  0.86 ms by infusing adenophostin-A (red symbols, *n* = 15). ISI values for corresponding spikes under these two conditions are statistically different (*p* < 0.01). Thus, intracellular Ca<sup>2+</sup> attenuates spike capacity at cortical GABAergic neurons.

Fig. 1C and D shows the effect of adenophostin-A on sequential spikes at cerebellar GABAergic Purkinje cells. The elevation of intracellular Ca<sup>2+</sup> appears to increase the number of spikes in a given time (Fig. 1C). The ISI values of spikes 1–2 to 4–5 are 15.59  $\pm$  0.72, 18.44  $\pm$  0.7, 19.37  $\pm$  0.75 and 20.22  $\pm$  0.9 ms under the control (blue symbols in Fig. 1D); and ISI's values are 13.57  $\pm$  0.6, 16.75  $\pm$  0.55, 17.52  $\pm$  0.56 and 18  $\pm$  0.7 ms in adenophostin-A (red symbols, *n* = 14). ISI values for corresponding spikes under these two conditions are statistically different (*p* < 0.01). Thus, intracellular Ca<sup>2+</sup> enhances spike capacity at cerebellar Purkinje cells.

### Mechanisms underlying the differential regulations of intracellular Ca<sup>2+</sup> to spike encoding at cortical and cerebellar GABAergic neurons

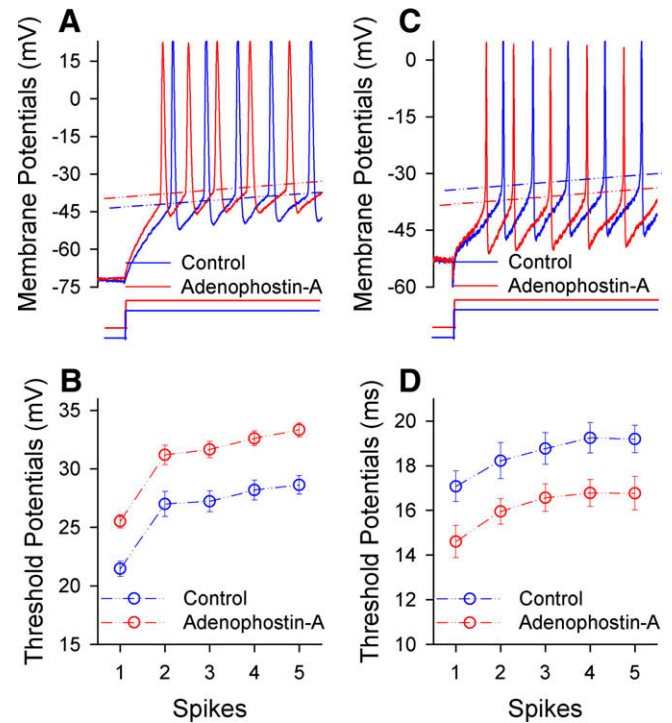
As action potentials are navigated by refractory periods and threshold potentials [15,22], the differential regulations of Ca<sup>2+</sup> to spike encoding at cerebellar and cortical GABAergic neurons are likely due to the influence of Ca<sup>2+</sup> on ARP and V<sub>t</sub>s differently. The changes in ARPs were measured by injecting depolarization pulses (3 ms) into the recording neurons after each of spikes. Threshold potentials are presented as the gap between the resting membrane potential (V<sub>r</sub>) and threshold potential (V<sub>t</sub>s).



**Fig. 2.** Intracellular  $\text{Ca}^{2+}$  prolongs refractory period at cortical GABAergic neurons and shortens ARP at cerebellar Purkinje cells in brain slices from FVB-Tg(Gad GFP)4570Swn/J mice. (A) The superimposed waveforms show ARP measures by changing the inter-pulse interval of depolarization currents (3 ms) under the control (red trace) vs. adenophostin-A infusion (dark-red trace) at cortical GABAergic neurons. (B) The comparisons of the ARP of sequential spikes under the control (red symbols) vs. adenophostin-A infusion (dark-red symbols) at cortical GABAergic neurons. (C) The superimposed waveforms show ARP measures by changing the inter-pulse interval of depolarization currents under the control (red trace) vs. adenophostin-A infusion (dark-red trace) at cerebellar GABAergic Purkinje cells. (D) The comparisons of the ARP of sequential spikes under the control (red symbols) vs. adenophostin-A infusion (dark-red symbols) at cerebellar Purkinje cells. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

Fig. 2 illustrates the influences of raising intracellular  $\text{Ca}^{2+}$  on ARPs at these two groups of neurons. At cortical GABAergic neurons, ARP values for spikes one to four are  $3.75 \pm 0.06$ ,  $4.16 \pm 0.09$ ,  $4.44 \pm 0.09$  and  $4.65 \pm 0.12$  ms under the control (red symbols in Fig. 2B); and ARPs are  $4.07 \pm 0.08$ ,  $4.49 \pm 0.12$ ,  $4.74 \pm 0.16$  and  $5.06 \pm 0.2$  ms by using adenophostin-A (dark-red symbols,  $n = 16$ ). ARP values for corresponding spikes under these two conditions are statistically different ( $p < 0.01$ ). At cerebellar GABAergic Purkinje cells, ARP values for spikes one to four are  $4.33 \pm 0.14$ ,  $5.1 \pm 0.24$ ,  $5.3 \pm 0.27$  and  $5.5 \pm 0.28$  ms under the control (red symbols in Fig. 2D); and ARPs are  $3.94 \pm 0.12$ ,  $4.5 \pm 0.17$ ,  $4.7 \pm 0.2$  and  $4.81 \pm 0.2$  ms in adenophostin-A (dark-red symbols,  $n = 15$ ). ARP values for corresponding spikes under these two conditions are statistically different ( $p < 0.01$ ). Thus, the elevation of intracellular  $\text{Ca}^{2+}$  prolongs ARPs of action potentials at cortical GABAergic neurons, and shortens ARPs at cerebellar Purkinje cells.

Fig. 3 shows the influence of raising cytoplasmic  $\text{Ca}^{2+}$  on Vts at these two groups of neurons. At cortical GABAergic neurons, the values of  $V_t - V_r$  for spikes one to five are  $21.46 \pm 0.65$ ,  $27 \pm 1.01$ ,  $27.22 \pm 0.89$ ,  $28.19 \pm 0.85$  and  $28.62 \pm 0.78$  mV under the control (red symbols in Fig. 3B); and its values are  $25.5 \pm 0.57$ ,  $31.2 \pm 0.82$ ,  $31.65 \pm 0.69$ ,  $32.6 \pm 0.62$  and  $33.33 \pm 0.6$  mV in adenophostin-A (dark-red symbols,  $n = 17$ ).  $V_t - V_r$  values for corresponding spikes under these two conditions are statistically different ( $p < 0.01$ ). At cerebellar Purkinje cells, The values of  $V_t - V_r$  for spikes one to five are  $17.1 \pm 0.69$ ,  $18.2 \pm 0.8$ ,  $18.76 \pm 0.71$ ,  $19.24 \pm 0.68$  and  $19.2 \pm$



**Fig. 3.** Intracellular  $\text{Ca}^{2+}$  raises threshold potentials at cortical GABAergic neurons and lowers  $V_t$ s at cerebellar Purkinje cells in brain slices from FVB-Tg(Gad GFP)4570Swn/J mice. (A) The superimposed waveforms show  $V_t$ s measures during burst spikes evoked by depolarization currents under control (red trace) vs. adenophostin-A infusion (dark-red trace) at cortical GABAergic neurons. (B) The comparisons of the  $V_t$ s of sequential spikes under control (red symbols) vs. adenophostin-A infusion (dark-red symbols) at cortical GABAergic neurons. (C) The superimposed waveforms show  $V_t$ s measures during burst spikes evoked by depolarization currents under the control (red trace) vs. adenophostin-A infusion (dark-red trace) at cerebellar Purkinje cells. (D) The comparisons of the  $V_t$ s of sequential spikes under the control (red symbols) vs. adenophostin-A infusion (dark-red symbols) at cerebellar Purkinje cells. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

$0.6$  mV under the control (red symbols in Fig. 3D); and its values are  $14.6 \pm 0.72$ ,  $15.95 \pm 0.57$ ,  $16.57 \pm 0.6$ ,  $16.78 \pm 0.61$  and  $16.76 \pm 0.75$  mV in adenophostin-A (dark-red symbols,  $n = 15$ ).  $V_t - V_r$  values for corresponding spikes under these two conditions are statistically different ( $p < 0.01$ ). Thus, the threshold potentials of action potentials are increased at cortical GABAergic neurons, and lowered at cerebellar Purkinje cells by raising intracellular  $\text{Ca}^{2+}$ .

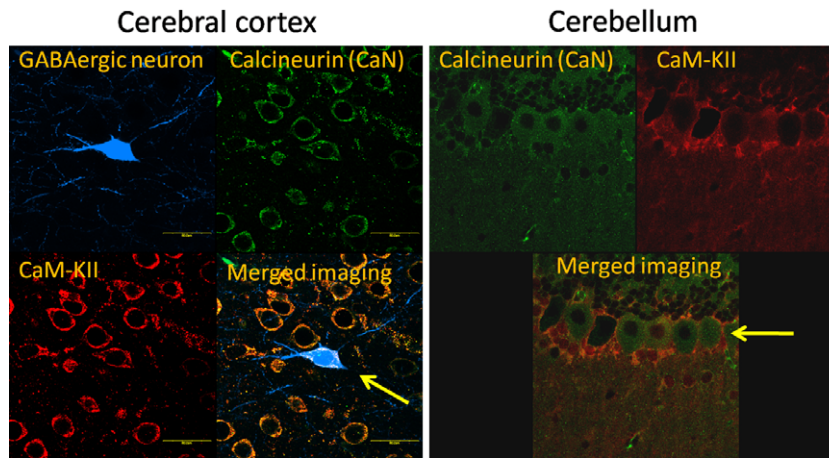
In summary, the different effects of intracellular  $\text{Ca}^{2+}$  on ARPs and  $V_t$ s at cortical GABAergic neurons and cerebellar Purkinje neurons explain why  $\text{Ca}^{2+}$  attenuates the spike encoding of cortical GABAergic neurons and enhances the function of Purkinje cells. In terms of the different influences of  $\text{Ca}^{2+}$  on neuronal intrinsic properties, we examined whether calcineurin and CaM-KII, the major targets of  $\text{Ca}^{2+}$  [26,27], are expressed differently at these two kinds of neurons.

Fig. 4 illustrates the double immuno-staining of CaN (green) and CaM-KII (red) in cerebral cortex (left panels) and cerebellum (right panels). Both CaN and CaM-KII are highly expressed at cortical GABAergic neurons. However, CaM-KII is lower at cerebellar Purkinje cells although CaN is well expressed. This result indicates that the ratios of CaM-KII to CaN are higher in cortical GABAergic neurons than cerebellar Purkinje cells, which may lead to the differential phosphorylation of VGSCs at these two kinds of neurons.

## Discussion

With studying the effects of intracellular  $\text{Ca}^{2+}$  on spike encoding, we found that cytoplasmic  $\text{Ca}^{2+}$  enhances the ability of spike





**Fig. 4.** The expression ratios of CaM-KII to calcineurin are higher at the cortical GABAergic neurons than cerebellar Purkinje cells in from FVB-Tg(Gad GFP)4570Swn/J mice. Left panels show the double immuno-staining of calcineurin (green) and CaM-KII (red) in cortical GABAergic neuron (cyan and pointed by yellow arrow in merged imaging). Right panels illustrate the immuno-staining of calcineurin (green) and CaM-KII (red) in cerebellar Purkinje cells (yellow arrow in the merged imaging) (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.).

encoding at cerebellar Purkinje cells and attenuates spike encoding at cortical GABAergic neurons (Fig. 1). Such different regulations in spike encoding are associated with the decreases of refractory periods and threshold potentials at cerebellar GABAergic Purkinje cells and the increases of these parameters at cortical GABAergic neurons (Figs. 2 and 3). In terms of the different regulations of cytoplasmic  $Ca^{2+}$  to ARP and Vts mediated by VGSC, we further found that the ratio of CaM-KII to calcineurin is higher at cortical GABAergic neurons than cerebellar Purkinje cells, which leads to the different levels of the phosphorylation of VGSCs at these two kinds of neurons.

Action potentials in the neurons activated by excitatory synaptic inputs raise the levels of cytoplasmic  $Ca^{2+}$  [28,29], which may cause plastic change in their activities. This change is expected to maintain neuronal homeostasis, stabilize precise neural encoding and coordinate neural functions (i.e., well-organized behaviors). Cerebellar Purkinje cells are believed to regulate the body balance in the motion. An enhancement of their encoding during neural activities will facilitate motion balance. However, the weakening of cortical GABAergic neurons during neural activity will confer excitatory neurons encoding the enriched messages for brain function being enhanced. Therefore, the different regulations of cytoplasmic  $Ca^{2+}$  to cerebellar Purkinje cells and cortical GABAergic neurons will strengthen the brain-controlled behaviors and cognition.

Cytoplasmic  $Ca^{2+}$  regulates the refractory period and threshold potential of action potentials, and in turn influences spike encoding at GABAergic neurons (Figs. 1–3). These results further support a notion that refractory periods and threshold potentials navigate neuronal encoding, which is concluded by examining a correlation between these parameters and spike encoding when raising the intensity of excitatory inputs, changing after hyperpolarization, and tracing postnatal development [15,22,23,25].

The ratios of CaM-KII to calcineurin are higher in cortical GABAergic neurons than cerebellar Purkinje cells. Therefore, CaM-KII will be dominantly activated when intracellular  $Ca^{2+}$  rises in cortical GABAergic neurons, which phosphorylates VGSCs to increase refractory periods and threshold potential of action potentials. On the other hand, calcineurin activity will be dominant when intracellular  $Ca^{2+}$  rises in cerebellar GABAergic Purkinje cells, which dephosphorylates VGSCs to decrease ARP and Vts. Our results and postulations are supported by a report that VGSCs are phosphorylated and inactivated by protein kinase C [30].

It is noteworthy that  $\alpha$ -subunit of CaM-KII and calcineurin have not been seen in hippocampal GABAergic neurons [31,32] although other reports disagree to this view [17,33,34]. Intracellular  $Ca^{2+}$  regulates the function of GABAergic neurons (Figs. 1–3). CaM-KII and calcineurin are more or less detected in these neurons (Fig. 4). Therefore, our results support the notion that  $Ca^{2+}$ /calmodulin signaling pathways are expressed and functional in GABAergic neurons in the brain [17].

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