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Short-Term Depression of Axonal Spikes at the Mouse Hippocampal Mossy Fibers and Sodium Channel-Dependent Modulation

Shunsuke Ohura[1] and Haruyuki Kamiya[1]

[1]*Department of Neurobiology, Hokkaido University Graduate School of Medicine, Sapporo, 060-8638, Japan*

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Corresponding should be addressed to Haruyuki Kamiya, M.D., Ph.D., Department of Neurobiology, Hokkaido University Graduate School of Medicine, Sapporo, 060-8638, Japan. Phone: 81-11-706-5027 Fax 81-11-706-7863. E-mail: kamiya@med.hokudai.ac.jp

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Author names and affilitations:

Shunsuke Ohura[1] and Haruyuki Kamiya[1]

[1]*Department of Neurobiology, Hokkaido University Graduate School of Medicine, Sapporo, 060-8638, Japan*

Author contributions:

H.K. designed the experiments and analysis. S.O. and H.K. performed the experiments. S.O. and H.K. performed the data analysis. H.K. wrote the manuscript. All the authors approved the final version of the manuscript.

Corresponding should be addressed to:

Haruyuki Kamiya, M.D., Ph.D.

Department of Neurobiology, Hokkaido University Graduate School of Medicine, Sapporo, 060-8638, Japan

Phone: 81-11-706-5027 Fax 81-11-706-7863

E-mail: kamiya@med.hokudai.ac.jp

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Abstract

Axonal spike is an important upstream process of transmitter release, which directly impacts on release probability from the presynaptic terminals. Despite the functional significance, possible activity-dependent modulation of axonal spikes has not been studied extensively, partly due to inaccessibility of the small structures of axons for electrophysiological recordings. In this study, we tested the possibility of use-dependent changes in axonal spikes at the hippocampal mossy fibers, where direct recordings from the axon terminals are readily feasible. Hippocampal slices were made from mice of either sex, and loose-patch clamp recordings were obtained from the visually identified giant mossy fiber boutons located in the stratum lucidum of the CA3 region. Stimulation of the granule cell layer of the dentate gyrus elicited axonal spikes at the single bouton which occurred in all or none fashion. Unexpected from the digital nature of spike signaling, the peak amplitude of the second spikes in response to paired stimuli at 50 ms interval was slightly but reproducibly smaller than the first spikes. Repetitive stimuli at 20 Hz or 100 Hz also caused progressive use-dependent depression during the train. Notably, veratridine, an inhibitor of inactivation

of sodium channels, significantly accelerated the depression with minimal effect on the initial spikes. These results suggest that sodium channels contribute to use-dependent depression of axonal spikes at the hippocampal mossy fibers, possibly by shaping the afterdepolarization (ADP) following axonal spikes. Prolonged depolarization during ADP may inactivate a fraction of sodium channels and thereby suppresses the subsequent spikes at the hippocampal mossy fibers.

Significance Statement

Spike signaling along axons is thought to highly reliable digital process. In this study, we tested the possibility of analog tuning of axonal spikes using direct recordings from single hippocampal mossy fiber terminals. We found that axonal spikes are subject to robust use-dependent short-term depression. Notably, the application of veratridine, an inhibitor of inactivation of sodium channels, selectively accelerates short-term depression with minimal effect on the initial axonal spikes. These results illustrate the novel form of short-term plasticity of axonal spikes in single axon terminal levels, and suggest that slow activating sodium channels of persistent-type (I_{NaP}) or resurgent types (I_{NaR}), different from the transient-type (I_{NaT}) responsible for spike generation, might be involved in modulation of paired-pulse depression of axonal spikes.

Introduction

Spike propagation along axon is highly reliable digital processes to carry neuronal information for a long distance without attenuation (Hodgkin and Huxley, 1952; Debanne, 2004; Kole and Stuart, 2012). Recent studies, however, have suggested that axonal spikes are regulated by the preceding neuronal activity or by the subtle changes in the local microenvironment due to influence from surrounding neuron and glia (Dugladze et al., 2012; Ruiz and Kullmann, 2012; Debanne et al., 2013; Kawaguchi and Sakaba, 2015). Frequency-dependent refractoriness at short intervals is well known examples of use-dependent regulation of axonal spikes due to intrinsic kinetic properties of ionic channels in axonal membrane. In addition, their excitability is also shown to be affected by transmitters released from the surrounding neuron and glia (Kamiya et al., 2002; Alle and Geiger, 2007; Sasaki et al., 2011; Uchida et al., 2012). Since hippocampal mossy fibers are unmyelinated axons readily accessible by these neuro- or glio-transmitters, and are suggested to express $GABA_A$, glycine, and kainate-type glutamate receptors on the axonal membranes (Kamiya and Ozawa, 2000; Schmitz et al., 2001; Ruiz et al., 2003; Kubota et al., 2010), it was expected that

97 activation of these axonal receptors may also contribute to regulate action
98 potential conduction and synaptic transmission at the mossy fiber-CA3
99 synapses.

100 Because of the reasonable size for direct electrophysiological recordings
101 from the large boutons of typically 3-5 µm in diameter (Geiger et al., 2002;
102 Bischofberger et al., 2006), hippocampal mossy fiber is studied intensively
103 not only for synaptic but also for axonal mechanisms. The new findings
104 obtained using the direct recording from the boutons include spike
105 broadening during repetitive stimuli (Geiger and Jonas, 2000), spike
106 amplification at *en passant* boutons (Engel and Jonas, 2005), passive
107 electrical signaling along the axon (Alle and Geiger, 2006), spike initiation at
108 the proximal axons (Schmitz-Hieber et al., 2008), and energy-efficient spike
109 generation (Alle et al., 2009).

110 On the other hand, activity-dependent modification of axonal spikes has
111 not studied extensively so far, except for use-dependent spike broadening of
112 action potentials during repetitive stimuli (Geiger and Jonas, 2000). They
113 showed robust use-dependent broadening of axonal spikes recorded from
114 mossy fiber terminals, and suggested that accumulated inactivation of

115 voltage-gated K^+ channels underlies this unique form of short-term
116 plasticity.

117 In the present study, we examined the possible use-dependent
118 modification of spike signaling along hippocampal mossy fibers,
119 unmyelinated axons with *en passant* structures typical for cortical axons. We
120 also explored the mechanisms underlying this novel form of short-term
121 plasticity, and addressed the possible contribution of afterdepolarization
122 (ADP), a hallmark of axonal spikes. Since ADP following axonal spike is
123 reported to be mediated by activation of resurgent-type Na^+ current at the
124 calyx of Held presynaptic terminals (Kim et al., 2010) and is robustly
125 enhanced by veratridine, an inhibitor of inactivation of Na^+ channel, we
126 tested if veratridine modulates use-dependent depression of axonal spikes.
127 Prominent use-dependent effect of veratridine suggests that sodium
128 channels play important roles not only in generation of axonal action
129 potentials, but also in modulating short-term plasticity by affecting ADP
130 following axonal action potentials.

131

Materials and Methods

Animals

C57BL/6J mice were initially purchased (Japan SLC, Hamamatsu, Japan) and later bred in-house. All animal procedures were performed in accordance with the Hokkaido University animal care committee's regulations. Every effort to minimize suffering and the numbers of animals was made throughout the study.

Preparation of hippocampal slices

Transverse hippocampal slices of 300 μm thick were prepared from C57BL/6J mice of either sex (p14-p43, number of animals = 58) as described previously (Shimizu et al., 2008) with some modifications. Animals were anesthetized with ether and the brain was dissected out in an ice-cold sucrose solution containing the following (in mM): 40 NaCl, 25 $NaHCO_3$, 10 glucose, 150 sucrose, 4 KCl, 1.25 NaH_2PO_4, 0.5 $CaCl_2$, and 7 $MgSO_4$ (Geiger et al., 2002). Transverse hippocampal slices were cut using a VT1200S microslicer (Leica Biosystems, Germany), and the above solution was

150 replaced with a NMDG-HEPES recovery solution containing the following
151 (in mM): 93 NMDG, 30 $NaHCO_3$, 25 glucose, 20 HEPES, 2.5 KCl, 1.2
152 NaH_2PO_4, 5 Na-ascorbate, 2 Thiourea, 3 Na-pyruvate, 0.5 $CaCl_2$, 10 $MgSO_4$,
153 and incubated for no longer than 15 min (Ting et al., 2014). Then, the
154 solution was exchanged again with artificial cerebrospinal fluid (ACSF)
155 containing the following (in mM): 127 NaCl, 1.5 KCl, 1.2 KH_2PO_4, 26
156 $NaHCO_3$, 10 glucose, 2.4 $CaCl_2$, and 1.3 $MgSO_4$, and the slices were kept in
157 an interface-type chamber saturated with 95% O_2 and 5% CO_2 at room
158 temperature (around 25°C).
159
160 **Electrophysiology**
161 The slices were perfused with the Ca^{2+}-free ACSF (equal concentration of
162 Mg^{2+} was replaced for Ca^{2+}; 0 $CaCl_2$ and 3.7 $MgSO_4$) at around 2 ml/min and
163 maintained at 24-26°C in a recording chamber. In addition, the slice surface
164 of the recording site was locally perfused with the above solution at about 0.2
165 ml/min through a flow pipe with a 250 µm open-tip diameter connected to an
166 electromagnetic valve system (Valve Bank; Automate Scientific, Berkeley,
167 CA, USA) for faster exchange of solution selectively around the recording

168 sites (Fig. 1*A*). The Ca^{2+}-free ACSF was used to suppress all synaptic
169 transmission and therefore eliminate possible recording from postsynaptic
170 neurons.

171 For extracellular recording of axonal spikes from single mossy fiber
172 boutons, glass pipettes containing the recording solution (typically 3~6 MΩ
173 electrode resistance) were placed on the visually-identified putative boutons
174 in the stratum lucidum under IR-DIC microscope (BX51WI, Olympus, Tokyo,
175 Japan), and gentle suction was applied to the recording pipettes. Loose patch
176 configuration was used to achieve less-invasive recording from the small
177 boutons for a long period. For instance, even under continuous focal
178 perfusion around the recoding site (see above, Fig. 1*A*), stable recordings for
179 long periods up to several hours are readily feasible, and therefore are suited
180 for quantitative pharmacological study of bath or focally applied drugs.

181 In experiments shown in Fig. 7, whole-cell current clamp recordings of
182 action potentials and ADP from the granule cell soma (Kamiya and Ozawa,
183 2000) were performed. Patch pipettes were filled with an internal solution
184 (pH 7.3) containing (mM): 140 potassium gluconate, 20 KCl, 0.2 EGTA, 2.0
185 $MgCl_2$, 10 Hepes, and 2.0 Mg-ATP. The resistance of the pipette was 4–8 MΩ

when filled with the internal solution. The access resistance was typically 15–30 MΩ immediately after obtaining whole-cell recordings, and was not allowed to vary by more than 20 % during the course of the experiment.

All recordings were made at room temperature (25 ± 1 °C), except in the experiments at more physiological temperatures (33 ± 1 °C) shown in red circles in Fig. 2*C*. Extracellular axonal spikes at mossy fiber boutons or intracellular action potentials at the granule cell soma were recorded with glass pipettes using a Multiclamp 700B amplifier (Molecular Devices, Sunnyvale, CA, USA). Signals were filtered at 10 kHz with 4-pole Bessel filter, sampled at 20 kHz, and analyzed offline with pCLAMP10 software (Molecular Devices, Sunnyvale, CA, USA).

Simulation

Simulated membrane potential (V_m) during axonal action potential at the hippocampal mossy fibers was calculated according to the Schmitdt-Hieber's model assuming "8 states" gating of sodium channels (accession: 128079, https://senselab.med.yale.edu/ModelDB/). This model is the latest revision of the mossy fiber model which is optimized for electrophysiological data

obtained by direct recording from axonal blebs (Schmidt-Hieber and Bischofberger, 2010). In this study, we carried out a simulation to validate the faithful monitoring of axonal spikes using this latest model.

Chemicals

Veratridine was purchased from Sigma-Aldrich (St. Louis, MO, USA). Tetrodotoxin was purchased from Funakoshi (Tokyo, Japan). All other chemicals were purchased from Wako Pure Chemical Industries (Tokyo, Japan).

Statistics

Data are expressed as the mean ± SEM, and n represents the number of recording boutons. Statistical analysis for comparison between the two paired groups were performed by Wilcoxon signed rank test, and P values of < 0.05 were accepted for significance. All statistical analyses were performed using R software (version 3.4.1)

Results

Recording of axonal spikes from single mossy fiber boutons

Axonal spikes elicited by stimulation of granule cell were directly recorded from a visually-identified mossy fiber boutons in mouse hippocampal slices (Fig. 1*A*). Electrical stimuli at the granule cell layer of dentate gyrus evoked the bi-phasic responses at the recorded boutons (Fig. 1*B*). These responses are likely to represent action potentials propagated along the mossy fiber axons, from the reasons mentioned below. First, these responses were evoked in all or none fashion in response to threshold stimuli (Fig. 1*C*). Second, focal application (Kamiya, 2012) of low concentration of tetrodotoxin (0.5 μM) abolished the responses (Fig. 1*D*), supported by the high level expression of voltage-dependent Na^+ channels at the mossy fiber boutons (Engel and Jonas, 2005).

It has been suggested that action potentials recorded extracellularly by loose-patch configuration well match with that of the first derivative of membrane potential (Meeks et al., 2005). To check for the adequate monitoring of axonal spikes in these recording conditions, we compared the

time course of the recorded axonal spike with that of the first derivative of simulated action potentials at the mossy fibers (Fig. 1*E*). For this purpose, we adopted the model assuming "8 states" gating of sodium channels at the hippocampal mossy fibers (Schmitdt-Hieber and Bishofberger, 2010). The time course of the recorded axonal spike is almost proportional to that of the first derivative of simulated action potential (dV_m/dt). All these suggested that the recorded responses are likely to represent adequately monitored axonal spikes from the single mossy fiber boutons.

Paired-pulse depression of axonal spikes

Then, we examined activity-dependent modulation of the axonal spikes. In response to paired stimuli at 50 ms interval, the peak amplitudes of second spikes were slightly, but reproducibly smaller than first spikes (Fig. 2*A*, 89.3 ± 0.9 % of control, n = 29, $P = 0.0000027$). To explore the time course of paired-pulse depression of axonal spikes, inter-stimulus intervals were varied from 50 to 500 ms (Fig. 2*B*). Depression were prominent at short intervals and almost recovered at 500 ms intervals (Fig. 2*C*; 89.8 ± 1.5, 92.6 ± 1.8, 97.1 ± 0.9, 102.1 ± 1.1 % of control at 50, 100, 200, and 500 ms intervals,

257 respectively; n = 9). These results showed that the paired-pulse depression
258 lasts for several hundreds of ms.

259 Recordings at room temperature may slow down channel gating and
260 thereby affect the degree and the time course of depression of axonal spikes.
261 To test for depression of axonal spikes at more physiological recording
262 temperature, similar experiments were carried out at 33 ± 1 °C. Substantial
263 depression still remained at intervals shorter than 200 ms (Fig. 2*C* red
264 circles; 87.7 ± 1.7, 93.2 ± 1.5, 98.2 ± 1.6, 100.4 ± 1.6 % of control at 50, 100,
265 200, and 500 ms intervals, respectively; n = 7). These results showed that the
266 paired-pulse depression occurs even at the physiological temperature.

267 Since the time course of the recorded axonal spike was expected to be
268 proportional to that of the first derivative of action potentials recorded
269 intracellularly, we reconstituted time course of membrane potential
270 transients by calculating the time-integral of axonal spikes recorded
271 extracellularly. As expected from inactivation of sodium channels by
272 prolonged depolarization during ADP, the peak amplitudes of time-integrals
273 of axonal spikes were reduced in responses to paired stimuli at 50 ms
274 intervals (to 94.4 ± 3.4 %, n = 20, $P = 0.01531$, Fig. 2D). We also measured

half-width of time-integrals of the extracellularly recorded axonal spike, although the difference was statistically not significant (to 104.2 ± 12.6 %, n = 20, $P = 0.7012$).

To confirm spike recordings from axon terminals, we used Ca^{2+} free solution to exclude the possibility of synaptically evoked spikes in postsynaptic cells. One may argue that recordings in Ca^{2+} free conditions may perturb observation in physiological conditions. To check for the roles of physiological concentration of Ca^{2+}, we examined the effect of application of Ca^{2+}-containing ACSF after establishment of recording in Ca^{2+}-free solution. Paired-pulse depression of axonal spikes was almost unaffected by application of Ca^{2+}-containing solution (Fig. 3*A*). The first (open circles) as well as second spikes (closed circles) were almost unchanged (to 102.3 ± 2.5 % and 104.7 ± 2.8 % of control, n = 8, Fig. 3*B, C*). The effects of Ca^{2+}-containing solution on the first and the second responses were statistically not different (P = 0.7422 and 0.4609, respectively). The waveform of the first and second axonal spikes were almost unchanged by Ca^{2+}-containing solution (Fig. 3*E*). These results indicate that paired-pulse depression of axonal spikes occurs in physiological condition.

293

294 **Suppression of paired-pulse depression by veratridine**

295 Since the time course of paired-pulse depression, as shown in Fig. 2, is
296 similar to that of afterdepolarization (ADP) which typically follows action
297 potential of hippocampal mossy fibers (Geiger and Jonas, 2000; Kamiya et al.,
298 2002), we supposed that paired-pulse depression are caused by progressive
299 inactivation of voltage-gated Na^+ channels (He et al., 2002) due to prolonged
300 ADP. To test this, veratridine, an inhibitor of inactivation of Na^+ channels,
301 was used, since the previous study at the calyx of Held axon terminals
302 showed that veratridine robustly enhances ADP (Kim et al., 2010). If ADP
303 was partly mediated by slowly activating Na^+ channels, possibly either
304 persistent type I_{NaP} (D'Ascenzo et al., 2009; Kole, 2011; Ghitani et al., 2016)
305 or resurgent type I_{NaR} (Raman and Bean, 1997), prolonged ADP may
306 inactivate significant fraction of transient Na^+ channels I_{NaT} responsible for
307 generation of action potentials in axons. As expected, focal application of 1
308 μM veratridine notably reduced the peak amplitude of the second spikes in
309 responses to the paired-stimuli at 50 ms interval, with minimal effects on the
310 first responses (Fig. 4*A*). The second spikes (closed circles) were reduced to

311 78.1 ± 1.8 % of control, whereas the first responses (open circles) were almost
312 unchanged (to 97.6 ± 1.8 % of control, n = 20, Fig. 4*B, C*). The effect of
313 veratridine on the first and the second responses was statistically significant
314 (P = 0.00000191). As a consequence, paired-pulse depression of axonal spikes
315 at 50 ms interval was enhanced by 1 μM veratridine (89.0 ± 1.1 % in control
316 and 71.9 ± 1.5 % during veratridine application, n = 20, P = 0.000001907, Fig.
317 4*D*). This use-dependent depression of axonal spikes by veratridine suggests
318 the notion that afterdepolarization are partly mediated by some types of
319 sodium channels with slow activation such as resurgent-type I_{NaR} or
320 persistent-type I_{NaP}. Prolonged depolarization during ADP by activation of
321 these slow activating sodium channels may inactivate a fraction of transient
322 sodium channels I_{NaT} responsible for generation of action potentials, and
323 thereby suppress the second spikes. Sustained depolarization at the time of
324 the second stimulus were also supported by the finding that the latency of
325 the second spike was shorter than the first spike (to 95.2 ± 0.9 %, n = 20, P =
326 0.000558). This also suggests that ADP plays a regulatory role on the second
327 spikes in response to paired-stimuli.
328

Veratridine accelerates depression of axonal spikes by repetitive stimuli

We also examined the effect of 1 μM veratridine on the responses to repetitive stimuli. First, we applied 20 Hz stimuli for 10 times, same 50 ms interval adopted for paired-pulse protocol in Fig. 4, to see if the effect is cumulative upon multiple stimuli. Veratridine caused the progressive decrease of the peak amplitude of axonal spikes (Fig. 5*A*) with minimal effect on the first responses. We also tested the effect of veratridine on the responses to high-frequency stimulation at 100 Hz for 1 s. Although axonal spikes faithfully followed in response to almost all stimuli even at 100 Hz, the amplitudes declined gradually during the train (Fig. 5*B*). Application of 1 μM veratridine again selectively suppressed the later responses with minimal effect on the first responses.

The 10th spikes during 20 Hz trains (closed circles) were reduced to 61.8 ± 3.1 %, whereas the first responses (open circles) were 98.8 ± 3.2 % of control (Fig. 5*C*, n = 13). The effects of veratridine on the 10th responses were different from those on the first spikes (P = 0.000244). The 100th spikes during 100 Hz trains (open circles) were reduced to 50.2 ± 8.2 %, whereas the first responses (closed circles) were 111.6 ± 4.8 % of control (Fig. 5*D*, n = 14).

347 The effect of veratridine on the first and the 100th responses was
348 statistically significant (P = 0.000366). These observations are consistent
349 with the prediction of inactivation of transient Na^+ channels I_{NaT} by
350 enhanced ADP due to application of veratridine.

351 In line with inactivation of I_{NaT}, not only amplitude but also latency and
352 duration of axonal spikes were prolonged during the train (Fig. 5*E*). The
353 latency to peak of the 10th spike during 20 Hz train was prolonged relative
354 to the 1st spike (to 102.6 ± 1.1 %, n = 13, P = 0.0002441) and further delayed
355 by veratridine (to 114.1 ± 2.4 %, n = 13, P = 0.0004883, Fig. 5*F*). Veratridine
356 also prolonged the duration of 10th spikes (151.2 ± 10.8 %) with minimal
357 effect on the first spikes (97.8 ± 2.1 %, n =13). The latency to peak of the
358 100th spike during 100 Hz train was prolonged compared to the 1st spike (to
359 128.2 ± 4.5 %, n = 14, P = 0.00109, Fig. 5*G*). These findings also support the
360 notions that cumulative inactivation of sodium channels underlies
361 use-dependent depression of axonal spikes.

362

363 **Tetrodotoxin partly restores paired-pulse depression of axonal spikes**

364 To further get insights into the activity-dependent tuning of the axonal

365 spikes, we also tested the effect of a sodium channel blocker tetrodotoxin
366 (TTX), since low concentration of TTX has been shown to suppress ADP at
367 the axon terminals of calyx of Held (Kim et al., 2010). Application of 50 nM
368 TTX suppressed the first and second spikes (Fig. 6*A*) to 66.4 ± 3.6 % and 70.1
369 ± 2.7 % (n = 9, Fig. 6*B, C*). Application of 20 nM TTX weakly suppressed the
370 first and second spikes to 90.8 ± 1.7 % and 92.8 ±1.9 % (n = 7, Fig. 6B, C). In
371 line with the notion that ADP may underlie depression of subsequent axonal
372 spikes, 50 nM TTX weakly, but significantly, restored paired-pulse
373 depression of axonal spikes (82.3 ± 2.5 % in control and 88.6 ± 2.5 % during
374 TTX application, n = 9, P = 0.03906, Fig. 6D).
375
376 **Veratridine enhances ADP in granule cell soma.**
377 The results so far support the notion that ADP-enhancement by
378 veratridine may cause much inactivation of I_{NaT} and thereby suppresses
379 subsequent axonal spikes in use-dependent manner. To further test this, we
380 recorded action potentials from the granule cell soma in the dentate gyrus,
381 which originates mossy fiber axons and exhibits pronounced ADP following
382 action potentials as in mossy fiber terminals (Geiger and Jonas, 2000). Brief

current injection (500-1000 pA, 1 ms) elicited action potentials followed by ADP (Fig. 7*A*). Application of 1 μM veratridine robustly enhanced and prolonged ADP (Fig. 7*A-C*) and caused multiple spiking by single stimuli, as similar to the calyx of Held axon terminals (Kim et al., 2010). Application of 1 μM veratridine enhanced the amplitude of ADP, as measured at 10 ms after the peak of action potentials (Fig. 7*B*), to 170.1 ± 14.7 % (n = 7, P = 0.01562, Fig. 7*C*). This finding is consistent with the previous study showing that resurgent sodium current I_{NaR} exists in granule cells (Castelli et al., 2007). Though the future study using whole-bouton recordings of ADP is clearly needed, we suppose that ADP in the mossy fiber terminals is mediated at least partly by voltage-dependent sodium channels, most likely I_{NaR}, and causes use-dependent depression of axonal spikes by cumulative inactivation of I_{NaT} by sustained depolarization during ADP.

The effect of 4-aminopyridine on the paired-pulse depression of axonal spike

Suppose that cumulative inactivation of I_{NaT} underlies depression of axonal spikes, blocking potassium channels may also modulate subsequent spikes by enhanced inactivation of I_{NaT} during the prolonged initial spike. To

check for the specificity of involvement of sodium channels, we tested the effect of potassium channel blocker in paired-pulse depression of axonal spikes. For this purpose, we adopted low concentration of 4-aminopyridine (4-AP), since action potentials in the mossy fiber terminal have been shown to broaden by slowing repolarization (Carta et al., 2014). Application of 10 µM 4-AP weakly reduced the amplitude of the first spikes to 86.1 ± 2.0 %, while the second spikes was strongly suppressed to 65.3 ± 5.2 % (n=9, P = 0.003906, Fig. 8*A-C*). As a consequence, paired-pulse depression of axonal spikes (to 80.3 ± 1.8 %, n = 9) was enhanced by 4-AP (to 61.6 ± 4.6 %, P = 0.003906, Fig. 8*D*) as similar to veratridine. It should be noted, however, that 4-AP strongly suppressed the negative deflection of axonal spikes (Fig. 8*A, E*), as expected from slowing of action potential repolarization. These findings suggest that 4-AP enhances paired-pulse depression in different ways from veratridine.

Discussion

In this study, we examined the possible activity-dependent tuning of axonal spikes recorded from single mossy fiber boutons in mouse hippocampal slices. We found that the axonal spikes display robust use-dependent depression in the physiological frequency ranges or even in the paired-stimuli condition. Notably, veratridine, an inhibitor of inactivation of Na^+ channels, prominently accelerated use-dependent depression with minimal effect on the first responses. All the results support that sodium channel-dependent mechanisms underlie modulation of use-dependent depression of axonal spikes at the mossy fibers.

Short-term depression of axonal spikes

Spike propagation along axons is highly reliable digital process which enables reliable information signaling in the central nervous system (Debanne et al., 2011). Recent studies, however, suggested that axonal spikes are subject to analogue modulation (Alle and Geiger, 2008; Debanne et al., 2013; Ohura and Kamiya, 2016). At hippocampal mossy fibers, one of the best studied axons in the central nervous system, activity-dependent

434 broadening of action potentials during high-frequency stimulation was
435 reported (Geiger and Jonas, 2000). It was suggested that accumulated
436 inactivation of voltage-dependent K^+ channels may slow repolarization phase
437 of action potential and thereby prolong the spike duration.

438 In this study, we found that the amplitude of axonal spikes recorded
439 extracellularly decreased in an activity-dependent manners. Paired-pulse
440 depression of spike amplitude lasted for several hundreds of ms (Fig. 2).
441 Since this time course closely related to that of ADP following action
442 potentials at the hippocampal mossy fibers (Geiger and Jonas, 2000; see also
443 Kamiya et al., 2002), we supposed that ADP may involve in the paired-pulse
444 depression of axonal spikes. Prolonged-depolarization during ADP may
445 inactivate a fraction of Na^+ channels, thereby suppress the subsequent
446 action potentials. Consistent with the prediction, delay in the peak of axonal
447 spikes became prominent during repetitive stimuli (Fig. 5*E*).

448 Detailed kinetic properties of Na^+ channels at the mossy fiber terminals
449 were examined previously (Engel and Jonas, 2005). They showed that
450 recovery from inactivation was substantially faster than that on the soma,
451 and almost fully recovered within 20 ms. Therefore, remaining inactivation

452 of Na^+ channels by preceding action potential might not be enough to cause
453 paired-pulse depression at 50 ms intervals, and we suppose that some
454 additional mechanisms, such as inactivation of Na^+ channels due to slow
455 afterdepolarization, may underlie depression of axonal spikes.

456 In this study, we adopted Ca^{2+} free conditions to exclude the possibility of
457 recordings of postsynaptic spikes. We wondered if this condition might affect
458 depression of axonal spikes. Therefore, we tested the effect of focal
459 application of Ca^{2+} containing solution to the recording sites, and confirmed
460 that depression was unchanged by this treatment (Fig. 3D).

461 It has been demonstrated that action potentials directly recorded from
462 axons of cerebellar Purkinje neurons in cultures show depression of peak and
463 occasionally conduction failure occurs during repetitive stimulation
464 (Kawaguchi and Sakaba, 2015). Alterations of spike amplitude and potential
465 involvement in failures have also been recorded in Purkinje cell axons in
466 slices (Khaliq and Raman, 2005; Rudolph et al., 2011). In sharp contrast,
467 axonal spikes are resistant to use-dependent depression at the calyx of Held
468 axon terminals which is specialized for high-fidelity signaling even at
469 high-frequency ranges (Sierksma and Borst, 2017). These results suggest

that frequency-dependent tuning of axonal excitability may be optimized for types of neuronal signals carried by various sorts of axon.

Sodium channel-dependent modulation of short-term depression

It was reported that veratridine, an inhibitor of inactivation of Na^+ channels, robustly up-regulates ADP, but not action potentials themselves, at the calyx of Held presynaptic terminals (Kim et al., 2010). We therefore supposed if veratridine may modulate depression of axonal spikes without affecting the initial action potentials. Consistent with the prediction, veratridine accelerated short-term depression of axonal spikes whereas the amplitude of the initial spikes was almost unaffected.

Voltage-gated sodium channels are categorized into three subtypes with different mode of activation, namely the transient type (I_{NaT}), the persistent type (I_{NaP}), and resurgent type (I_{NaR}). The I_{NaT} is essential for generation of action potential, though I_{NaP} and I_{NaR} are supposed to increase neuronal excitability and regulate burst firings (Raman and Bean, 1997; D'Ascenzo et al., 2009; Kole, 2011). Since veratridine is a rather non-specific inhibitor of inactivation of sodium channels, it may exert facilitative actions on all of I_{NaT},

488 I_{NaP} and I_{NaR}.

489 Involvement of sodium channels was also suggested by the findings as
490 below. A low concentration of sodium channel blocker TTX at 50 nM slightly,
491 but significantly restored paired-pulse depression of axonal spikes, as shown
492 in Fig. 6. We also tested for 20 nM TTX, which was reported to suppress ADP
493 at the calyx of Held axon terminals (Kim et al., 2010). TTX at 20 nM weakly
494 suppressed the amplitude of first spike, although PPD of axonal spikes was
495 not affected significantly. We could not explain the reason why PPD was not
496 affected by 20 nM TTX significantly. The difference in preparation (e.g. calyx
497 of Held vs. mossy fiber axon terminals) or experimental conditions may
498 explain the minimal effect of 20 nM TTX. Alternatively, additional
499 mechanisms independent of activation of sodium channels, e.g. capacitative
500 components of propagating action potentials may mediate ADP at least in
501 part (Kim et al., 2010).

502 On the other hand, potassium channel blocker 4-AP also enhanced
503 paired-pulse depression as veratridine, although the waveform of axonal
504 spikes was changed significantly, as expected from slowing of repolarization
505 by 4-AP (Carta et al., 2014). Taken together, our results suggest the

506 involvement of sodium channels, rather than potassium channels, in ADP
507 and short-term depression of axonal spikes.

508 To account for the selective effects on short-term depression of axonal
509 spikes with minimal effect on initial action potential, we considered as
510 follows. Hippocampal mossy fiber terminals express high-density of Na^+
511 channels which amplify action potentials (Engel and Jonas, 2005), and
512 secure safe conduction over multiple boutons which are expected to be a risk
513 factor for conduction failure due to impedance mismatch of thin axons and
514 large boutons. Highly abundant expression of sodium channels suggests that
515 a fractional enhancement of I_{NaT} may not modify the amplitude of action
516 potentials caused by regenerative processes substantially. In other words,
517 the facilitative effect of veratridine on I_{NaT}, if any, may not become obvious on
518 the peak amplitude of action potentials. In any case, the selective modulation
519 by veratridine revealed sodium channel-dependent mechanisms, possibly
520 through enhancing I_{NaP} or I_{NaR}, underlay short-term depression of axonal
521 spikes.

522 In this study, our observations were limited in loose-patch clamp
523 recordings, since they are less invasive and stable for prolonged periods, and

524 thereby it was possible to show whole time course of the veratridine effect
525 including washout. In support of our interpretation, ADP recorded from the
526 granule cells in dentate gyrus was shown to be enhanced and prolonged by
527 veratridine (Fig. 7). Since granule cells express resurgent sodium current
528 I_{NaR} (Castelli et al., 2007), veratridine activated I_{NaR} and enhanced ADP.
529 Similar mechanisms in the mossy fiber terminals may account for the
530 accelerated depression of axonal spike observed in this study. To directly
531 assess the mechanisms, however, whole-bouton recording would help in
532 getting the mechanistic insights.

533 Veratridine robustly enhanced and prolonged ADP recorded from the
534 granule cells in the dentate gyrus, and caused multiple spiking by single
535 stimuli (Fig. 7). It was speculated that much stronger effect of veratridine on
536 somatic ADP might be resulted either from different modes of AP stimulation
537 or different recording configurations (i.e. whole cell recordings vs.
538 non-invasive cell attached recordings).

539 It would be also helpful to perform numerical simulations by modelling
540 study. To date, several realistic models of action potentials have been
541 proposed in hippocampal mossy fibers (Engel and Jonas, 2005; Alle et al.,

2009; Schmidt-Hieber and Bischofberger, 2010). All these previous simulations do not incorporate afterdepolarization in their models. It is obvious that the revised model incorporating afterdepolarization after identifying the ionic mechanisms by future experiments.

Subcellular recordings from axon terminals

In this study, we established direct recordings from the single axon terminals of the hippocampal mossy fibers, following stimulation of their originating soma (granule cells in dentate gyrus). This approach will offer unique opportunities to study spike signaling along axons, since hippocampal mossy fibers consist of *en passant* structures typical for many cortical axons. It should be mentioned that recent studies clarified many important notions on the heterogeneity of ionic conductances (Debanne et al., 2013; Kole and Stuart, 2012) as well as local control by the influence of microenvironment. Ectopic spiking of axon terminals (Dugladze et al., 2012) may be the important subject of future studies.

Functional implications

Despite functional importance, use-dependent short-term plasticity of spike signaling has not been studied intensively. Slight but reproducible depression even at paired-pulse conditions, as observed in this study, suggest the physiological significance of this form of analog tuning. Since modification of axonal spikes has strong impact on transmitter release and plasticity by affecting entry of Ca^{2+} into the presynaptic terminals (Geiger and Jonas, 2000; Kawaguchi and Sakaba, 2015), we suggest that short-term depression of axonal spikes may play an important modulatory role in short-term plasticity at the mossy fiber-CA3 synapses. Further studies with whole-bouton recordings will clarify the detailed mechanisms of this novel presynaptic form of plasticity.

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688
689

690 **Figure Legends.**

691

692 **Figure 1.** Loose-patch clamp recordings of axonal spikes from the single
693 mossy fiber boutons. ***A***, Schematic drawing of experimental arrangement.
694 Stimulating electrode was placed in the granule cell layer of the dentate
695 gyrus, and the evoked responses were recorded from visually-identified
696 single mossy fiber boutons. Surrounding region of the recording site was
697 focally perfused with a continuous flow of perfusate through a flow-pipe. A
698 photograph showing IR-DIC image of the recorded bouton (arrow). ***B***,
699 Representative traces of the axonal spikes recorded from the single mossy
700 fiber boutons. ***C***, All-or-none feature of the axonal spikes, which appear
701 above the threshold stimulus intensity (0.2 mA in this recording). ***D***, Effect of
702 focal application of tetrodotoxin (TTX) at 0.5 μM. ***E***, Comparison of the time
703 course of simulated dV_m/dt and the recorded axonal spikes. Simulated
704 membrane potential (V_m) during axonal action potential was calculated
705 according to the latest model of action potentials at mossy fibers (see
706 Materials and Methods). In the right panel, the recorded axonal spike in ***B***
707 was superimposed with the first derivative of simulated V_m (dV_m/dt, middle

708 panel).

709

710 **Figure 2.** Paired-pulse depression (PPD) of axonal spikes recorded from
711 single mossy fiber boutons. ***A***, At 50 ms interval, the amplitude of the second
712 spike (closed circle) was slightly reduced than the first spike (open circle). ***B***,
713 Superimposed traces of paired-pulse responses at 50, 100, 200 and 500 ms
714 intervals. ***C***, Time course of PPD of axonal spikes recorded at 25 ± 1 ℃ are
715 shown in black circles (n = 9). Data of similar experiments recorded at 33 ±
716 1 ℃ are also shown in red circles (n = 7). ***D***, Time-integrated traces of
717 axonal spikes recorded extracellularly by loose-patch clamp configuration,
718 which are supposed to reflect intracellular membrane potential changes
719 during action potentials, in response to the first (open circle) and second
720 stimuli (closed circle, blue trace). Note that superimposed traces in the right
721 panel show reduction of the peak amplitudes.

722

723 **Figure 3.** Minimal effects of Ca^{2+}-containing (Ca^{2+}(+)) ACSF on axonal spikes.
724 ***A***, Focal application of Ca^{2+}(+) ACSF to the surrounding area of the recorded
725 boutons (see Fig. 1*A*) exhibited no clear effects on the 1st (open circles) and

2nd (closed circles) axonal spikes. ***B***, Time course of the amplitude of the first and second spikes during Ca^{2+}(+) ACSF application. ***C***, Summary data of the effect of Ca^{2+}(+) ACSF on the first (open bar) and second spikes (closed bar, n = 8). ***D***, Summary data of paired-pulse depression (PPD) of axonal spikes in control condition (open bar) and in Ca^{2+}(+) ACSF. ***E***, Superimposed traces of the first (open circle) and 2nd spike (closed circle) in the control condition and in Ca^{2+}(+) ACSF.

Figure 4. Selective modulation of PPD of axonal spikes by veratridine, an inhibitor of inactivation of sodium channels. ***A***, Focal application of 1 μM veratridine selectively suppressed the amplitude of the second spike (closed circle) with minimal effect on the first spike (open circle). ***B***, Time course of the amplitude of the first and second spikes during veratridine application (n = 20). ***C***, Summary data on the effect of veratridine on the first (open bar) and second spikes (closed bar, n = 20, ** $P < 0.01$). ***D***, Summary data of paired-pulse depression (PPD) of axonal spikes in control condition (open bar) and in the presence of veratridine (closed bar). ***E***, Superimposed traces

744 of the first (open circle) and 2nd spike (closed circle) in control condition and
745 in the presence of veratridine.
746
747 **Figure 5.** Veratridine-induced use-dependent modulation of axonal spikes
748 during repetitive stimuli. ***A***, ***B***, Veratridine at 1 μM minimally affected the
749 first spike (open circle), while selectively reduced the subsequent responses
750 to 20 Hz (***A***) and 100 Hz (***B***) trains . ***C***, ***D***, Time course of the first, 10th or
751 100th spikes during veratridine application (n = 13 and 14, respectively). ***E***,
752 Superimposed traces of the first (open diamond) and 100th spike during 100
753 Hz train (closed diamond) in the control condition and in the presence of
754 veratridine. ***F***, ***G*** Summary data of latency of the first and the last spikes
755 during 20 Hz (***F***) and 100 Hz trains (***G***). The 100th spikes during 100 Hz
756 trains in the presence of veratridine (***G***) were reduced in size substantially,
757 and therefore were unable to measure the latency quantitatively.
758
759 **Figure 6.** Effect of low concentration of tetrodotoxin (TTX) on axonal spike. ***A***,
760 TTX at 50 nM partially suppressed both the first (open circle) and second
761 spike (closed circle). ***B***, Time course of the amplitude of the first and 2nd

762 spikes during application of 20 nM and 50 nM TTX (n = 7 and 9, respectively)
763 as shown by open and closed diamonds and circles, respectively. ***C***, Summary
764 data of the effect of 20 nM and 50 nM TTX on the first (open bar) and second
765 spikes (closed bar) ***D***, Paired-pulse depression was weakly restored by 50 nM
766 TTX (closed bar, n = 9, ** $P < 0.01$), while 20 nM TTX did not affect
767 significantly (n = 7). ***E***, Superimposed traces of the first (open circle) and
768 second spike (closed circle) in control condition and in the presence of 50 nM
769 TTX.
770
771 **Figure 7.** Veratridine potentiated afterdepolarization (ADP) in the granule
772 cell soma. ***A***, Veratridine at 1 μM strongly enhanced ADP following action
773 potentials elicited by brief current injection into the dentate granule cells,
774 which originates the mossy fiber axons. Prolonged ADP during veratridine
775 application was often accompanied by multiple spikes after current injection.
776 ***B***, Expanded time course of action potential and ADP. ADP amplitude was
777 quantified at 10 ms after the peak of the action potential (arrowhead). ***C***,
778 Summary data on the effect of veratridine on the ADP amplitude (n = 7, ** P
779 < 0.01).

Figure 8. Effect of low concentration of 4-aminopyridine (4-AP) on axonal spike. ***A***, 4-AP at 10 μM partially suppressed both the first (open circle) and second spike (closed circle). ***B***, Time course of the amplitude of the first and 2nd spikes during 4-AP application (n = 9). ***C***, Summary data of the effect of 4-AP on the first (open bar) and second spikes (closed bar). ***D***, Paired-pulse depression was enhanced by 4-AP (closed bar, n = 9, ** $P < 0.01$). ***E***, Superimposed traces of the first (open circle) and second spike (closed circle) in control condition and in the presence of 4-AP. Note that the negative peak of axonal spike was strongly suppressed while the positive peak was weakly affected, suggesting 4-AP may slow the decaying phase of axonal action potentials.