The involvement of persistent Na\(^+\) current in spike-initiation
in primary sensory neurons of the rat mesencephalic trigeminal nucleus

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ABSTRACT

It has recently been shown that the persistent Na\(^+\) current (\(I_{\text{NaP}}\)) is generated in the proximal axon in response to somatic depolarization in neocortical pyramidal neurons, while the involvement of \(I_{\text{NaP}}\) in spike-initiation is still unclear. Here we show a potential role of \(I_{\text{NaP}}\) in spike-initiation of primary sensory neurons in the mesencephalic trigeminal nucleus (MTN) that display a backpropagation of the spike initiated in the stem axon toward the soma in response to soma depolarization. Riluzole (10 µM) and tetrodotoxin (10 nM) caused an activation delay or a stepwise increase in the threshold for evoking soma spikes (S-spikes) without affecting the spike itself. Simultaneous patch-clamp recordings from the soma and axon hillock (AH) revealed that bath application of 50 nM tetrodotoxin increased the delay in spike activation in response to soma depolarization, leaving the spike-backpropagation time from the AH to soma unchanged. This indicates that the increase in activation delay occurred in the stem axon. Furthermore, under a decreasing intracellular concentration gradient of QX-314 from the soma to AH created by QX-314-containing and QX-314-free patch pipettes, the amplitude and maximum rate of rise (MRR) of AH-spikes decreased with an increase in the activation delay following repetition of current pulse injections, while S-spikes displayed much less decreases in amplitude and MRR. This suggests that comparing to S-spikes, AH-spikes more accurately reflect the attenuation of axonal-spike by QX-314, consistent with the spike-backpropagation nature. These observations strongly suggest that low-voltage-activated \(I_{\text{NaP}}\) is involved in spike-initiation in the stem axon of MTN neurons.
INTRODUCTION

The persistent or non-inactivating Na\(^+\) current (I\(_{\text{NaP}}\)) has extensively been investigated in neocortical pyramidal neurons (Crill 1996). Given the expression of I\(_{\text{NaP}}\) in the dendrites or soma, the I\(_{\text{NaP}}\) is considered to play roles in the forward communication between the dendrites and soma (Chance et al. 2002), and considered to control the membrane excitability in the soma-dendrite in the voltage region just subthreshold for spike generation (Crill 1996). However, the upregulation of I\(_{\text{NaP}}\) by PKC caused a decrease in the threshold for spike activation in neocortical pyramidal neurons (Astman et al. 1998), in which spikes are initiated in the proximal axon (Stuart and Sakmann 1994). More recently, the local application of TTX to the proximal axon of neocortical layer V pyramidal neurons has been shown to block the I\(_{\text{NaP}}\) recorded in the soma in response to soma depolarization, suggesting that I\(_{\text{NaP}}\) is primarily generated in the proximal axon (Astman et al. 2006). Nevertheless, there is still no explicit evidence that spikes are initiated by the activity of I\(_{\text{NaP}}\) in the proximal axon. On the other hand, a transient or inactivating and low-voltage-activated Na\(^+\) current was found to be expressed in the axon far beyond the initial segment (IS) of neocortical layer V pyramidal neurons (Colbert and Pan 2002), and blockade of those inactivating Na\(^+\) channels by locally applied tetrodotoxin (TTX) caused an increase in the threshold for spike activation (Colbert and Johnston 1996). Thus, it is still unclear whether I\(_{\text{NaP}}\) is directly involved in the initiation of spikes in the proximal axon.

The primary sensory neurons supplying periodontal mechanoreceptors or jaw-closer muscle spindles are unique in generating spikes in response to synaptic inputs onto the somata (Verdier et al. 2004), thereby displaying two distinct types of spikes, one arising from sensory organs and the other caused by somatic inputs (Saito et al. 2006). This is because their somata are exceptionally located in the mesencephalic trigeminal nucleus (MTN) within the brainstem subsequently receiving various synaptic inputs (Hinrichsen and Larramendi
1970; Liem et al. 1992). We also demonstrated previously that MTN neurons display spike-backpropagation from the spike-initiation site somewhere in the stem axon to the soma in response to injection of current pulses into the soma, and suggested that somatic inputs or impulses arising from sensory organs, whichever trigger spikes in the stem axon first, can be forwarded to their target synapses (Saito et al. 2006). Therefore, we aimed to elucidate the role of $I_{\text{NaP}}$ in spike-initiation in the stem axon of MTN neurons by using a dual patch-clamp recording method. In the present study, we have found that the process of spike initiation in the stem axon is highly sensitive to riluzole, 10 nM TTX and QX-314, strongly suggesting an involvement of $I_{\text{NaP}}$ in spike-initiation in MTN neurons.
METHODS

Whole-cell recordings

The whole-cell patch-clamp recording method was essentially similar to that used in our previous studies (Kang et al. 2004; Saito et al. 2006). Briefly, coronal slices of 200–250 µm thickness were made from the brainstem of Wistar rats (6–18 days postnatal). The standard extracellular solution had the following composition (in mM): 124 NaCl, 26 NaHCO₃, 1.8 KCl, 1.2 KH₂PO₄, 2.5 CaCl₂, 1.3 MgCl₂, and 10 glucose. The internal solution of the patch pipettes had the following ionic composition (in mM): 123 K-gluconate, 18 KCl, 10 NaCl, 3 MgCl₂, 2 ATP-Na₂, 0.3 GTP-Na₃, 10 HEPES; pH 7.4 adjusted with KOH for whole-cell recordings. The membrane potential values given in the text were corrected for the junction potential (10 mV) between the internal solution for the whole-cell recording (negative) and the standard extracellular solution. The recording chamber with a volume of 1.0 ml was continuously perfused with the extracellular solution at a flow rate of 1.0–1.5 ml/min.

Using Axopatch 1D, 200A, 200B, and MultiClamp 700A (Molecular Devices, Foster City, CA), single or dual whole-cell current-clamp recordings were made on MTN neurons viewed under Nomarski optics (BX-50WI, Olympus, Tokyo, Japan). Two patch pipettes used for simultaneous whole-cell recordings were pulled using the same parameter configuration (P-97, Sutter Instruments, Novato, CA) and their pipette resistances were 4–6 MΩ. Since all experiments were carried out on the recordings in which the series resistance was less than 10 MΩ, Axopatch 200A/B was used in the normal current-clamp mode, consequently causing no practical difference in the current-clamp performance between Axopatch 1D and 200A/B (Saito et al. 2006). Since the series resistance compensation was disabled in the current-clamp mode of Axopatch 1D and 200A or not used in Axopatch 200B, only the recordings that showed no sign of apparent bridge imbalance were included in the
analysis. Moreover, because spikes were always triggered after the offset of current pulses, an inappropriate bridge balance, if any, would not affect the spike height. All recordings were made at room temperature (21–24 °C). Records of currents and voltages were low-pass filtered at 5–10 kHz (3-pole Bessel filter), digitized at a sampling rate of 40 kHz (Digidata 1322A, Molecular Devices) and stored on a computer hard disk.

**Stimulation and drug application**

With a tungsten microelectrode (impedance; 1 MΩ at 5 kHz), microstimulation (intensity; 0.5–5.0 µA, duration; 60 µs) was applied to the stem axon at a site 40–60 µm apart from the soma. Riluzole (Sigma-Aldrich, St. Louis, MO), tetrodotoxin (TTX, Wako Pure Chemical, Osaka, Japan) and 4-aminopyridine (4-AP, Sigma-Aldrich) were bath-applied at concentrations of 10–20 µM, 10–50 nM and 0.2–0.5 mM, respectively. The membrane-impermeable lidocaine analogue, QX-314 (Sigma-Aldrich) was included in the internal solution at a concentration of 0.5–5 mM.

**Subcellular application of QX-314 by a dual whole-cell recording method**

In order to apply QX-314 locally to a subcellular region, we performed a dual whole-cell recording using QX-314-containing and QX-314-free patch pipettes. The QX-314-containing patch pipette was placed on the soma of an MTN neuron, and the QX-314-free patch pipette on the AH whose patch membrane was ruptured first. This would create a decreasing concentration gradient of QX-314 from the soma toward the AH. This was simulated with Lucifer Yellow (LY, Sigma-Aldrich). One patch pipette filled with the internal solution containing 0.1 % LY was placed on the soma of an MTN neuron, and the other patch pipette filled with the LY-free internal solution was placed, not right on the AH site, but on a site near the AH (Fig. 1). This is because the possible mechanical distortion of the AH membrane by the patch pipette may prevent LY from diffusing into the axon if the
patch pipette is placed right on the AH site. A steady-state concentration gradient decreasing from the soma to the AH was achieved 5–7 min after the establishment of the dual whole-cell (DWC) recording (Fig. 1A). The fluorescent intensity was higher around the LY-containing soma pipette (a) while it was much lower around the LY-free pipette placed near the AH (b). However, 2 min after the removal of the AH pipette from the MTN neuron, the LY fluorescence spread homogenously all over the soma, and subsequently revealed the stem axon (*, Fig. 1B). Thus, it was assumed that similar to LY, a steady-state concentration gradient of QX-314 would also be achieved by establishing a dual whole-cell recording. This would be useful to examine the subcellular localization of QX-314-sensitive channels.

Data given in the text are presented as mean ± SD unless otherwise mentioned and statistical significance was assessed using the two-tailed t-test and one-way ANOVA.
RESULTS

Increased delay in spike activation by riluzole and low concentration of TTX

In our previous study (Saito et al. 2006), simultaneous patch-clamp recordings from the soma and axon hillock (AH) revealed a spike-backpropagation from the spike-initiation site in the stem axon to the soma through the AH in response to injection of a short current pulse into the soma. Soma spikes (S-spikes) emerged with a delay after the offset of the short current pulse (Fig. 2), partly due to the backpropagation and partly due to the electrotonic separation from the current pulse injection site in the soma to the spike-initiation site in the stem axon. As shown in Fig. 2Aa, with an increase in the peak voltage level ($v$, inset) of depolarizing responses to current pulses, the S-spikes was triggered with less delay. If $I_{NaP}$ is involved in the spike-initiation in MTN neurons, the activation of S-spikes would be further delayed by inhibiting $I_{NaP}$ with selective blockers, riluzole or a low concentration of TTX (Schwindt and Crill 1996; Stafstrom et al. 1985). Therefore, we first examined if riluzole or a low concentration of TTX causes a further delay in spike-initiation in response to injection of current pulses into the soma, without affecting the spike itself.

When the time to the maximum rate of rise ($T_{MRR}$) of the S-spikes measured from the timing of the current pulse offset was plotted against the peak voltage level ($v$, arrow) attained at the end of current pulses (inset, Fig 2Aa), $T_{MRR}$ decreased with an increase in $v$ (black circles, Fig. 2Ac and Ad), as reported previously (Saito et al. 2006). Bath application of 10 µM riluzole increased the threshold for evoking S-spikes with the same delay ($T_{MRR}$) (inset, superimposed traces *1 in Fig. 2Aa and Ab), and caused a greater delay in response to the same depolarization ($v$) (inset, superimposed traces *2 in Fig. 2Aa and Ab). Thus, riluzole shifted the $v$-$T_{MRR}$ relationship positively (compare black and red circles, Fig. 2Ac and Ad), without attenuating the spike amplitude markedly (compare interrupted lines in Fig. 2Aa and Ab). These observations suggest that $I_{NaP}$ is directly involved in spike-initiation.
This could be more clearly seen in the presence of 4-aminopyridine (4-AP), which revealed a ramp-like depolarization preceding the S-spike (Fig. 2Ba). Due to this ramp-like depolarization, S-spikes could be triggered with much longer delays. Such ramp-like depolarization appeared to be mediated by \( I_{NaP} \) (Schwindt and Crill 1996; Stafstrom et al. 1985). Indeed, 10 nM TTX attenuated the ramp-like depolarization markedly (Fig. 2Ba and Bb), and shifted the \( v-T_{MRR} \) relationship positively by 8.7 ± 2.3 mV (\( n = 4 \), Fig. 2Bc and Bd) in the presence of 0.2–0.5 mM 4-AP, when the two \( v \) values, having almost the same \( T_{MRR} \) measured before and after TTX application (0.59 ± 0.13 ms and 0.59 ± 0.12 ms, respectively), were compared. Similarly, 10 \( \mu \)M riluzole also shifted the \( v-T_{MRR} \) relationship positively by 9.3 ± 1.8 mV (\( n = 4 \)) in the presence of 0.2–0.5 mM 4-AP, when the two \( v \) values, having almost the same \( T_{MRR} \) measured before and after riluzole application (0.59 ± 0.09 ms and 0.58 ± 0.09 ms, respectively), were compared. In these pooled data, MTN neurons in which the S-spike attenuated by more than 6% of its control amplitude by TTX or riluzole were not included. S-spikes were attenuated only by 4.7 ± 0.9% and 4.4 ± 0.6% of their control amplitudes by 10 nM TTX and 10 \( \mu \)M riluzole, respectively. Thus, a stepwise increase in the threshold for or a prolongation of the delay in the activation of S-spikes without marked changes in amplitude was seen following the application of these blockers, suggesting an involvement of \( I_{NaP} \) in the initiation of S-spikes. In the next experiments, we performed a dual whole-cell recording in order to examine which processes during the spike-initiation and -backpropagation were affected by TTX.

**Differential sensitivity to TTX between soma and axonal spikes**

Simultaneous patch-clamp recordings were obtained from the soma and AH to investigate whether such an activation delay or threshold increase occurred at the cell body or at the stem axon where the axonal spike is initiated. S- and AH-spikes were evoked by each
one of the three kinds of electrical stimulation applied every 10 sec in the following order: current pulse injection into the soma (S1) and that into the AH (S2), and stimulation of the stem axon (S3) as schematically illustrated in Fig. 3A. As partly shown in Fig. 3Ba, during the perfusion of 50 nM TTX for 350 sec, both the simultaneously recorded S- and AH-spikes evoked by current pulse injection into the AH (and soma; figure not shown) gradually decreased in amplitude as well as in the maximum rate of rise (MRR) (Fig. 3Ea and Eb). Similarly, the S- and AH-spikes arising from the invasion of axonal spikes following stimulation of the stem axon decreased in amplitude (Fig. 3Bb). These decreases in amplitude and MRR were accompanied by the parallel increases in the activation delay of the AH- and S-spikes in response to injection of current pulses (*1 and *3 in Fig. 3Ba). Simultaneously, the spikes caused by axonal stimulation also markedly increased in latency (*2 in Fig. 3Bb). Ten seconds after such a delayed activation in response to the current pulse injection (*3 in Fig. 3Ba), even a stimulation of the stem axon with intensities up to 1.25 times the threshold intensity failed to evoke “antidromic” S- and AH-spikes, leaving no apparent depolarization (*4 and *6 in Fig. 3Bb). After the failure of spike generation in the stem axon seen exactly 350 sec after the TTX application, the current pulse injection into the AH also failed to evoke spikes (*5 in Fig. 3Ba).

However, the S- and AH-spikes could still be evoked (*7 in Fig. 3Ca) when the stimulus current intensity was increased (compare the voltage levels reached at the pulse end, upper panels in Fig. 3Ba and Ca). The normalized amplitude and MRR almost continuously decreased with time during the 50 nM TTX perfusion (Fig. 3Ea and Eb). There appeared no marked differences between the two normalized amplitudes and between the two normalized MRRs obtained just before and after blockade of the axon-originated spikes (Fig. 3Ea and Eb), whereas $T_{MRR}$ sharply increased just before the blockade of the axonal spike (Fig. 3Ec), and the threshold for evoking the S- and AH-spikes apparently increased just after blockade.
of the axonal spike (compare the subthreshold depolarization levels indicated by horizontal arrows in Fig. 3Ba and Ca). This activation delay and the subsequent threshold increase are consistent with the positive shift of the \(v-T_{\text{MRR}}\) relationship following the application of riluzole or TTX (Fig. 2).

Provided that there are no differences in the kinetics, density and TTX sensitivity among \(\text{Na}^+\) channels distributed in the soma, the AH and the stem axon, it is possible that the invasion of the axonal spike into the AH would fail most easily during the progress of the blockade of \(\text{Na}^+\) channels because the threshold increase would be the greatest due to the large capacitative load of the large cell body. If this is the case, the AH pipette should record some subthreshold response after the possible blockade of the spike invasion. However, there was no subthreshold response in the AH recording (see traces *4 and *6 in Fig. 3Bb and Cb), indicating that the blockade of the axonal spike is not due to invasion failure. Instead, the marked prolongation of the antidromic latency (see traces *2 in Fig. 3Bb) suggests that the 50 nM TTX first suppressed the activation of \(\text{Na}^+\) channels at the site to which the stimulation was applied, consequently causing a conduction delay and complete blockade of axonal spikes. In fact, this marked prolongation of the antidromic latency occurred simultaneously with the increase in \(T_{\text{MRR}}\), which sharply increased from 0.46 ± 0.13 to 1.03 ± 0.16 ms by 0.58 ± 0.11 ms (\(n = 5\)) just before the abolishment of the axonal spike (Fig. 3Ec). In spite of the lack of marked differences in the peak amplitude between the spikes obtained in response to current pulse injections just before and after the blockade of the axonal spike (compare traces *3 and *7 in Fig. 3Ba and Ca), the current pulse depolarization for evoking spikes has to be increased in a stepwise manner by 13.8 ± 1.3 mV (\(n = 5\)) immediately after the failure of spike generation in the stem axon (compare the voltage levels at the pulse-end indicated by horizontal arrows in Fig. 3Ba and Ca). This value may be slightly overestimated, because these stronger depolarizations activated spikes
with a shorter \( T_{MRR} \) (2.39 ± 0.18 ms) than that of spikes seen just before the activation failure (2.63 ± 0.24 ms). This observation is consistent with a previous report, in which a similar threshold increase following abolishment of the axonal spikes with local application of TTX was observed in subicular pyramidal cells that display the spike-initiation in the proximal axon in response to soma depolarization (Colbert and Johnston 1996). In view of such a spike-initiation mechanism, the present observations would indicate that the lower threshold axonal spike is more sensitive to TTX than the higher threshold S-spike. Therefore, it is likely that the activation delay of the S-spike increased with the progress of the blockade of \( \text{Na}^+ \) channels in the stem axon, and the threshold for activation of the S-spike increased in a stepwise manner just after the complete blockade of the axonal spike initiation. This possibility was further analyzed in the next experiment.

**Delayed spike-initiation by TTX disclosed by dual patch-clamp recording from the soma and AH**

The zero-crossing time and the rise time of the first time derivative of the S-spike were denoted as \( T_{on-S} \) and \( T_{r-S} \), which represent the onset latency and the reciprocal of the rate of regenerative process of the S-spike, respectively (Fig. 4A). Then, the \( T_{MRR} \) of the S-spike \( (T_{MRR-S}) \) corresponds to the sum of \( T_{on-S} \) and \( T_{r-S} \). In our previous study, the spike-backpropagation in MTN neurons was well revealed by the simultaneous whole-cell current-clamp and cell-attached voltage-clamp recordings from the soma and AH, respectively (Fig. 1 in Saito et al. 2006). A depolarizing current pulse injection into the soma first generated the axonal spike, which in turn backpropagated to sequentially trigger AH- and S-spikes (Fig. 4B, and also see Fig. 1 in Saito et al. 2006). Since the initiated axonal spike is well reflected in the onset of the S- or AH-spikes due to the electrotonic continuity between the stem axon and the soma, the \( T_{on-S} \) reflects the latency to the axonal
spike generation \((T_{on-A} \leq T_{on-AH} \leq T_{on-S}, \text{ Fig. 4A and B})\), while the \(T_{r-S}\) primarily reflects the time for the regenerative process of the S-spike \((\Delta T_S \leq T_{r-S}, \text{ Fig. 4A and B})\). The possible differential effects of TTX on the \(\text{Na}^+\) channels distributed across the soma and the stem axon were evaluated by analyzing these parameters: \(\text{MRR, } T_{\text{MRR}}, \Delta T_S, T_{on}\) and \(T_r\).

When the \(T_{\text{MRR}}\) was plotted against the decrease in the normalized MRR, the \(T_{\text{MRR}}\) increased almost linearly with a decrease in the normalized MRR, before and after blockade of the axonal spikes (Fig. 4Ca). However, the difference \((\Delta T_S)\) between the timing of the MRR of the AH-spike and that of the S-spike \((T_{MRR-S} - T_{MRR-AH})\) remained almost constant before the blockade of the axonal spikes (Fig. 4Cb), indicating that the spike-backpropagation time from the AH to the soma did not change appreciably in spite of the delayed emergence of S-spikes. This suggests that the delay occurred primarily at the stem axon. Therefore, it is likely that the blockade of \(\text{Na}^+\) channels by TTX progressed first in the stem axon without markedly affecting the soma and AH. This possibility was further explored by quantifying the respective processes during the spike initiation and its backpropagation.

When the threshold for evoking the axonal spike is increased by the progress of the blockade of \(\text{Na}^+\) channels in the stem axon (Hille 1968), the delay in the axonal spike generation would increase with an increase in the threshold for evoking axonal spikes. This is because the threshold increase and the spike generation delay occur in parallel when spikes are triggered from potentials changing along the rising envelope of the subthreshold underlying depolarization. Then, the \(T_{on}\) increase primarily reflects the threshold increase for evoking axonal spikes, consistent with the positive shift of the \(v-T\) relationship following the application of TTX and riluzole (Fig. 2). By contrast, the \(T_r\) increase may reflect the decrease in the rate of the regenerative process of spikes in the soma. Before the blockade of the axonal spike, the increase in \(T_{\text{MRR}}\) was more accurately reflected in the increase in the \(T_{on}\) than in the \(T_r\) (Fig. 4Cc and Cd), suggesting an increase in the threshold for evoking the
axonal spike with the progress of the blockade of Na\(^+\) channels in the stem axon (see DISCUSSION), which could lead to the failure of axonal spike generation. Therefore, the increase in the \(T_{MRR}\) before the blockade of axonal spikes is completely consistent with the simultaneous prolongation of the antidromic latency due to a decrease in the excitability in the stem axon. Following the application of 50 nM TTX, the \(T_{on}\) increased in S-spikes from 0.37 ± 0.27 to 0.83 ± 0.17 ms by 0.46 ± 0.09 ms \((n = 5)\), and similarly increased in AH-spikes from 0.29 ± 0.22 to 0.60 ± 0.31 ms by 0.30 ± 0.12 ms \((n = 5)\). There was no significant difference \((p > 0.9)\) in the ratio of the \(T_{on}\) increase to the \(T_{MRR}\) increase between S-spikes (83 ± 9%) and AH-spikes (83 ± 4%).

After the blockade of axonal spikes, the increase in \(T_{MRR}\) was almost equally reflected in the increase in the \(T_{on}\) and the \(T_r\) (Fig. 4Cc and Cd). Since there is no spike-backpropagation after blockade of axonal spikes, the parallel increases in the \(T_{on}\) and \(T_r\) may simply be due to the threshold increase in the soma spike and the slow down of the regenerative process in the generation of the soma spike, respectively, presumably following the progress of the blockade of Na\(^+\) channels expressed on the soma membrane with larger MRR decreases. Thus, a large \(T_{on}\) increase accompanied by a small \(T_r\) increase occurred following an initial small MRR decrease until the complete blockade of the axonal spike, while smaller parallel increases in \(T_{on}\) and \(T_r\) occurred following a large MRR decrease after blockade of axonal spikes, suggesting a differential effect of TTX on the increase in the threshold between the axonal and S-spike. The \(T_{on}\) increase during a 10% decrease in MRR was more than 2 times larger in the S-spike (0.21 ± 0.06 ms) recorded before than in that (0.09 ± 0.02 ms) recorded after the blockade of axonal spikes and/or the stepwise threshold increase, following application of 50 nM TTX in 5 MTN neurons examined. Thus, it is likely that, in terms of the threshold increase measured as the \(T_{on}\) increase, the TTX-sensitivity is more than two times higher in Na\(^+\) channels presumably expressed on the
axon than in those expressed on the soma.

**Differential effects of QX-314 on S- and AH-spikes**

Since the axonal spike appeared to be most sensitive to a low concentration of TTX, the involvement of $I_{\text{NaP}}$ in the initiation of spikes in the stem axon was further examined in the next experiments by using QX-314, to which $I_{\text{NaP}}$ is more sensitive than the rapidly inactivating $I_{\text{Na}}$ (Schwindt and Crill 1996; Stafstrom et al. 1985). Considering the nature of spike-backpropagation, the attenuation of axonal spikes generated in the stem axon should be more accurately reflected in AH-spikes than in S-spikes. Therefore, it was examined whether AH-spikes are more sensitive to QX-314 than S-spikes are. Dual whole-cell recordings were made from the soma and AH of single MTN neurons; and only one of the two patch pipettes contained 0.5 mM QX-314. When the degree of attenuation is simply larger in the spike recorded by the QX-314-containing pipette than in that simultaneously recorded by the QX-314-free pipette, it would not be possible to determine whether QX-314 sensitive $I_{\text{NaP}}$ is highly expressed only at the site of the QX-314 pipette or evenly expressed across the two recording sites. In order to prove the higher expression of $I_{\text{NaP}}$ on the AH, it must be examined under the condition of a decreasing concentration gradient of QX-314 from the soma to AH whether the degree of spike attenuation is larger in AH-spikes than in S-spikes or not. Therefore, we placed one pipette containing 0.5 mM QX-314 internal solution on the soma and the other pipette containing normal internal solution on the AH, whose patch membrane was ruptured first (see METHODS).

Alternate injections of current pulses into the soma and AH every 10 sec were started under a presumed steady gradient of the QX-314 concentration reached 5–7 minutes after establishing the dual whole-cell recordings (see METHODS). Intracellular injection of 0.5 mM QX-314 did not apparently affect the holding potential and the apparent membrane time
constant. However, with repetition of the current pulse injection, the AH-spike decreased in amplitude sharply while the amplitude of the S-spike remained almost constant, irrespective of the current pulse injection site as seen in the superimposed traces (Fig. 5Aa and Ab), and in the plot of the normalized spike amplitude of the AH- and S-spikes against the time after rupture of the membrane patch of the QX-314-containing pipette ($p < 0.001$, ANOVA, Fig. 5Ba). Moreover, the normalized MRR of the respective spikes decreased more promptly in AH-spikes than in S-spikes ($p < 0.001$, ANOVA, Fig. 5Bb). In 4 MTN neurons examined (Fig. 5Ca and Cb), both the normalized peak amplitude and MRR of AH-spikes (peak amplitude, $0.79 \pm 0.14$; MRR, $0.48 \pm 0.13$) obtained 20 to 25 min after rupture of the membrane patch of the 0.5 mM QX-314-containing pipette were significantly ($p < 0.05$, ANOVA) smaller than those of S-spike (peak amplitude, $0.95 \pm 0.02$; MRR, $0.64 \pm 0.12$). Thus, in spite of the decreasing concentration gradient of QX-314 from the soma toward the AH, the AH-spike was more markedly attenuated than the S-spike. Similar observations were made in 6 MTN neurons, in which either 0.5 mM ($n = 4$) or 1 mM QX-314 ($n = 2$) was injected through the soma pipette. However, with the 5 mM QX-314-containing pipette ($n = 5$), both the S- and AH-spikes disappeared after a few abortive spikes were evoked, in response to current pulse injections that caused depolarization to a level more positive than 0 mV (figure not shown), indicating no apparent contamination of Na$^+$ spikes by Ca$^{2+}$ spikes, as reported previously in MTN neurons (Yoshida and Oka 1998). Therefore, the backpropagated S-spike is likely to be much less sensitive to QX-314 than the AH-spike. These observations indicate a larger involvement of $I_{NaP}$ in the AH-spike than in the S-spike, presumably due to a larger reflection of axonal spikes in the AH-spike than in the S-spike.

In order to examine if the larger attenuation of the AH-spike in comparison with the S-spike by QX-314 is due to a larger reflection of the axonal spike attenuation, the relationship between the activation delay ($T_{MRR}$ or $T_{on}$) and the MRR decrease following
repetitive spike activation in the presence of intracellular QX-314 was compared between S- and AH-spikes. Depending on the level of \( v \) examined, the increase in \( T_{MRR} \) and \( T_{on} \) varied (Fig. 5Da and Db). Since the \( T_{MRR} \) appeared to be proportional to the inverse of \( v \), a certain threshold increase causes a larger increase in \( T_{MRR} \) or \( T_{on} \) when examined at a lower \( v \) rather than at a higher \( v \) (Fig. 2). Consistent with the case of TTX, the \( T_{MRR} \) increase was more accurately reflected in the \( T_{on} \) increase than in the \( T_r \) increase (Fig. 5Da and Db), suggesting that the spike-initiation delay in the stem axon was due to the QX-314 blockade of Na\(^+\) channels distributed in the stem axon. There was no significant difference in the ratio of the \( T_{on} \) increase to the \( T_{MRR} \) increase between S- and AH-spikes. However, when the decrease in MRR was plotted against the increase in \( T_{MRR} \), the decrease in MRR per unit increase in \( T_{MRR} \) was invariably and significantly (\( p < 0.02 \)) larger in AH-spikes (open circles) than in S-spikes (filled circles), regardless of the injection site, whether the soma or the AH, of the current pulses (Fig. 5Dc and Dd). As largely and almost equally reflected in \( T_{on} \) increase, both the increases in the \( T_{MRRs} \) of S- and AH-spikes would indicate the excitability decrease in the stem axon and/or the attenuation of possible axonal spikes. Then, it is likely that such an attenuation of the possible axonal spike was reflected by the MRR decrease more accurately in AH-spikes than in S-spikes, consistent with the nature of spike-backpropagation that the initiated spike in the stem axon should be more accurately reflected in AH-spikes than in S-spikes. Therefore, it is strongly suggested that the larger attenuation of AH-spikes in comparison with S-spikes was largely due to the blockade of Na\(^+\) channels distributed in the stem axon by QX-314, even under the condition of the decreasing concentration gradient of QX-314 from the soma toward the AH.
DISCUSSION

Activation delay in spike-backpropagation

MTN neurons, having round somata, displayed the backpropagation of the axonal spike to the soma through the AH. The nature of the active backpropagation is well reflected in the amplitude and rate of rise of the backpropagated S-spikes, which are larger than those of the preceding AH-spikes (Figs. 1 and 2 in Saito et al. 2006), differing from that seen in cortical pyramidal cells (Stuart and Sakmann 1994). This unusual backpropagation suggests that the threshold for evoking spikes is higher in the soma than in the stem axon in spite of the abundant presence of Na⁺ channels in the soma membrane as revealed by a larger MRR in S-spikes than in AH-spikes. The application of Na⁺ channel blockers, TTX, riluzole and QX-314, commonly caused a marked activation delay in such spike-backpropagation. The dual patch-clamp recordings disclosed that the activation delay was increased due to the blockade of Na⁺ channels in the stem axon.

Then, how did Na⁺ channel blockade in the stem axon increase the activation delay? Due to the electrotonic separation between the soma and the stem axon in MTN neurons, the membrane depolarization evoked in the soma membrane would spread into the stem axon, reaching the peak value at least more than 0.5 ms after the peak of the soma depolarization as revealed in the dual whole-cell recordings from the soma and AH (see Fig. 2B in Saito et al. 2006). Provided that axonal spikes are triggered from the threshold potentials shifting positively along the rising time course of the subthreshold depolarization in the stem axon due to a progressive blockade of Na⁺ channels in the stem axon, the degree of prolongation in the activation delay of S-spikes is dependent on the electrotonic distance between the soma and the spike-initiation zone and on the degree of the blockade of Na⁺ channels in the stem axon. In spinal motoneurons, a similar delay in the generation of low-voltage-activated Na⁺ current responsible for spike-initiation in the initial segment was seen in response to a voltage
command applied to the soma under a voltage-clamp condition (Araki and Terzuolo 1962). Thus, the prolongation of the activation delay of S-spikes without changing its shape suggests a partial blockade of Na⁺ channels in the spike initiation zone that is electrotonically remote from the soma.

**Differential sensitivities to TTX and QX-314 between the transient and persistent Na⁺ currents**

It has been reported in CA1 hippocampal neurons that the IC₅₀ values for the effect of TTX on the persistent and transient Na⁺ currents are approximately 9 and 37 nM, respectively, while 1 µM lidocaine reduced the $I_{NaP}$ to 24% of the control, leaving the transient Na⁺ current almost unchanged (Hammarstrom and Gage 1998). In the present study, riluzole and 10 nM TTX increased the onset delay and the threshold of the S-spike without changing its shape (Fig. 2), suggesting that the blockade of $I_{NaP}$ progressed selectively in the stem axon. During the perfusion of 50 nM TTX, with a decrease in the MRR, the onset delay increased two times more sharply in S-spikes before the blockade of the axonal spike than in those evoked with stronger current pulses after the blockade, presumably reflecting the difference in the threshold increase between the possible axonal spike and S-spike (Fig. 4Cc and Cd). This may be consistent with the difference in the IC₅₀ for TTX between the persistent and transient Na⁺ currents. Furthermore, the dual whole cell recordings using QX-314-containing and QX-314-free patch pipettes revealed that the MRR decrease with an increase in the activation delay was significantly larger in AH-spikes than in S-spikes (Fig. 5D). Considering the nature of spike-backpropagation in MTN neurons, this observation strongly suggests that the presumed axonal spike is highly sensitive to QX-314 in comparison with S-spikes. Thus, the differential sensitivities to riluzole, TTX and QX-314 between the possible axonal spike and the S-spike strongly suggest that MTN neurons express different
Na⁺ channels in the stem axon and the soma, and that the stem axon of MTN neurons expresses $I_{\text{NaP}}$.

**Involvement of the transient vs. persistent Na⁺ currents in spike-initiation**

It has been reported in CA1 hippocampal pyramidal neurons that Na⁺ channels involved in the initiation of spikes are located in the axon, 30–60 µm away from the soma, and have an activation threshold lower by 7–8 mV than those of Na⁺ currents or spikes examined in the soma membrane (Colbert and Johnston 1996). A similar observation was also made in neocortical pyramidal neurons (Colbert and Pan 2002). Such a difference in the activation threshold of Na⁺ currents between the proximal axon and soma membrane is consistent with the results of a classical study on motoneurons under a voltage-clamp condition where IS and soma-dendritic spikes were mediated by two distinct Na⁺ currents having lower and higher activation thresholds, respectively (Araki and Terzuolo 1962). However, these Na⁺ currents having a lower activation threshold appeared to be transient, but not persistent.

The $I_{\text{NaP}}$ is known to have an activation threshold lower by about 10 mV than the transient or fast inactivating one (Crill 1996; Stafstrom et al. 1985). The upregulation of $I_{\text{NaP}}$ by PKC caused a decrease in the threshold for spike activation in neocortical pyramidal neurons (Astman et al. 1998), suggesting that $I_{\text{NaP}}$ is involved in spike-initiation. Furthermore, the local application of TTX to the axon of neocortical layer V pyramidal neurons has been shown to abolish the $I_{\text{NaP}}$ recorded in the soma, suggesting that $I_{\text{NaP}}$ is primarily generated in the proximal axon of neocortical pyramidal layer V neurons (Astman et al. 2006). Since the axon including IS of retinal ganglion cells was found to densely express the sodium channel Na_v1.6, this channel was assumed to be responsible for initiating impulses in the axon (Boiko et al. 2003). However, the kinetics of Na_v1.6 examined in the
recombinant expression system was distinct from that of the $I_{NaP}$ (Dietrich et al. 1998; Smith et al. 1998). Nevertheless, it is of interest that in Purkinje neurons obtained from ataxic mice lacking the expression of Na$_{1.6}$, persistent and resurgent Na$^+$ currents were markedly reduced in comparison with transient Na$^+$ current (Raman and Bean 1997; Raman et al. 1997). Taken together, as demonstrated in the present study, the spike-initiation at the stem axon is likely to be mediated by the lower-threshold and persistent type of Na$^+$ channel that is highly sensitive to riluzole, TTX and QX-314.
GRANTS

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FIGURE LEGENDS

FIG. 1. Subcellular concentration gradient of LY under a dual whole-cell recording.

A: A fluorescence photomicrograph showing the decreasing concentration gradient of Lucifer Yellow (LY) from the soma toward the AH created by establishing the dual whole-cell recordings from the soma and a site near the AH of an MTN neuron by using two patch pipettes containing LY (a) and normal internal solution (b), respectively. A superimposed image of four photomicrographs taken at four different depths of focus, 5 min after the establishment of the dual whole-cell (DWC) recording, showing the location of the two patch pipettes in relation with the soma of the MTN neuron. Note the weaker fluorescent intensities in the deeper sections around the LY-free near AH pipette during the DWC mode.

B: Two min after the removal of the AH pipette from the MTN neuron, the LY fluorescence spread homogenously all over the soma and consequently revealed stem axon (*).

FIG. 2. Prolongation of the delay in spike activation by riluzole and a low concentration of TTX.

A: Soma spikes (S-spikes) evoked by current pulse injection into the soma before (black traces, a) and after the application of 10 µM riluzole (red traces, b). The $v$ and $T_{MRR}$ represent the potential level reached at the pulse-end and the time to the maximum rate of rise (MRR) of the S-spikes measured from the timing of the current pulse offset, respectively (inset, a). Horizontal interrupted lines in (a) and (b) indicate the peak level of the S-spike with the minimal $T_{MRR}$ obtained before riluzole application. Traces *1 in (a) and (b) and traces *2 in (a) and (b) were superimposed (inset, b). Note the threshold increase for evoking S-spikes (*1) and the increase in $T_{MRR}$ (*2) by riluzole. Plotting of $T_{MRR}$ against $v$ on a linear (c) and a logarithmic scale (d), before (black circles) and after riluzole application (red circles). Voltage and time calibrations in (a) also apply in (b). Note a positive shift of
the $v$ and $T_{MRR}$ relationship by riluzole.

**B:** S-spikes evoked by current pulse injection into the soma before (black traces, $a$) and after application of 10 nM TTX (red traces, $b$) in the presence of 4-AP. Note the ramp-like depolarization preceding the S-spike and the long delay in triggering S-spikes ($a$). Horizontal interrupted lines in ($a$) and ($b$) indicate the peak level of the S-spike with the minimal $T_{MRR}$ obtained before TTX application. The traces indicated with arrows in ($a$) and ($b$) were superimposed in inset ($b$) to show an increase in the threshold for evoking S-spikes with the same delay by TTX (inset, $b$). Also note the marked attenuation of the ramp-like depolarization by TTX. Plotting of $T_{MRR}$ against $v$ on a linear ($c$) and a logarithmic scale ($d$), before (black circles) and after the application of TTX (red circles) in the presence of 0.2 mM 4-AP. Voltage and time calibrations in ($a$) also apply in ($b$).

**FIG. 3.** Differential sensitivity to TTX between axonal and soma spikes.

**A:** A schematic diagram showing the arrangement of the recording and stimulating sites. A dual whole-cell recording was made from the soma and AH of an MTN neuron, and the electrical stimulation was applied to the stem axon. The current pulse injection into the soma (S1) and that into the AH (S2), and stimulation of the stem axon (S3) were sequentially applied every 10 sec. $T_{MRR-S}$ and $T_{MRR-AH}$ represent the activation time of S-spike and AH-spike.

**B:** Superimposed sample traces of AH-spikes (upper panels, $a$ and $b$) and those of S-spikes (lower panels, $a$ and $b$) during application of 50 nM TTX for the initial 350 sec. Simultaneous recordings of AH- and S-spikes ($a$) evoked by current pulses applied to AH, and of AH- and S -spikes ($b$) evoked by stimulation of the stem axon. Horizontal arrows in ($a$) indicate the peak potential levels caused by the current pulse injection into the AH. Horizontal interrupted lines in ($a$) indicate the peak levels of the AH- and S-spikes denoted
with *3. In order to minimize artifacts included in the responses to stimulation of the stem
axon, a response to a subthreshold stimulation of the stem axon was subtracted from all the
responses shown in (b).

C: Superimposed sample traces of simultaneously recorded AH-spikes (upper panels, a and
b) and S-spikes (lower panels, a and b), obtained more than 360 sec after the application of
50 nM TTX. AH- and S-spikes (a) were evoked by injection of the stronger current pulse
into AH, while the stimulation of the stem axon with 1.25 times the threshold intensity no
longer evoked AH- and S-spikes (b). Horizontal arrows in (a) indicate the same potential
levels as shown in (Ba). A response to a subthreshold stimulation of the stem axon was
subtracted from all the responses shown in (b). Traces labeled with *1 to *7 were obtained
sequentially in response to sequential stimulation (S1–S3) applied every 10 sec.

D: Sample traces of the temporal derivatives of the simultaneously recorded AH- (a) and
S-spikes (b) evoked by current pulse injection into the soma. Note that the timing of MRR
of the AH-spike (open arrowhead) preceded that of the S-spike (filled arrowhead) evoked in
response to soma depolarization.

E: Plotting of the peak amplitude (a) and MRR (b) normalized to their control, and of the
$T_{MRR}$ (c), of AH- and S-spikes (open and filled circles, respectively), against the time during
perfusion of 50 nM TTX. X marks on the x-axis denote the failures of the axonal spike. In
spite of the continuous decrease in the normalized amplitudes (a) and in the normalized
MRRs (b), before and after blockade of the axonal spike, $T_{MRR}$ increased sharply just before
the blockade of the axonal spike (c).

FIG. 4. Effects of TTX on spike initiation delay.

A: The first derivative of the simultaneously recorded AH- and S-spikes evoked in response
to current pulse injection into the soma. $T_{MRR-S}$ is composed of $T_{on-S}$ and $T_{r-S}$, which
represent the onset latency and the reciprocal of the rate of the regenerative process of the S-spike, respectively.

B: A schematic diagram showing the whole process of spike-backpropagation. The latency to spike-initiation ($T_{on-A}$) and the time required for spike-backpropagation along the stem axon ($\Delta T_A$) and between the AH and soma ($\Delta T_S$) were denoted in relation to $T_{on-S}$ and $T_{r-S}$.

C: Plotting of $T_{MRR}$ of S- (filled circles, $a$) and AH-spikes (open circles, $a$), and plotting of $\Delta T_S$ ($= T_{MRR-S} - T_{MRR-AH}$) of S- (filled triangles, $b$) and AH-spikes (open triangles, $b$), obtained in response to the two constant current pulses before and after the blockade of axonal spikes (gray column), against the decrement of the MRR normalized to the control. Note the constant backpropagation time from the AH to the soma in spite of the sharp increase in the total backpropagation time, indicating the spike-initiation delay in the stem axon. Plotting of $\Delta T_{on-S}$ and $\Delta T_r$ of S-spikes (filled triangles and diamonds, respectively, $c$) and AH-spikes (open triangles and diamonds, respectively, $d$), obtained before and after blockade of axonal spikes (gray column), against the decrement of the normalized MRR to the control. Note the sharp $\Delta T_{on}$ increase before the blockade of axonal spikes, in comparison with that after the blockade.


A: Superimposed sample traces of AH- and S-spikes (left and right panels, respectively, in $a$ and $b$). AH- and S-spikes were simultaneously recorded by using two patch pipettes containing normal and 0.5 mM QX-314 internal solution, respectively. AH- and S-spikes ($a$) and those ($b$) evoked in response to injection of current pulses into the AH and soma, respectively. Voltage and time calibrations in ($a$) also apply in all other panels.

B: Plotting of the spike amplitude ($a$) and MRR ($b$) normalized to the controls of the AH-spikes (open circles) and S-spikes (filled circles), partly shown in ($A$), against the time
after rupture of the patch membrane of the QX-314-containing soma pipette. Both the spike amplitude and the MRR decreased more sharply in AH-spikes than in S-spikes, in spite of decreasing concentration gradient of QX-314 from the soma to AH \( (p < 0.001, \text{ANOVA}) \).

C: The mean ± SD values of the normalized amplitude \((a)\) and MRR \((b)\) of the AH- (open circles) and S-spikes (filled circles), obtained 20–25 min after the establishment of dual whole-cell patch-clamp in 4 MTN neurons. The control was obtained 5–7 min after dual whole-cell recording. *: \( p < 0.05 \) (ANOVA).

D: Plotting of the increments of \(T_{\text{MRR}}\) (circles), \(T_{\text{on}}\) (triangles), and \(T_{r}\) (diamonds) value of the AH-spikes evoked by current injection into the AH \((a)\), and those of the S-spikes evoked by current injection into the soma \((b)\), against the decrement of normalized MRR of respective spike. Note that \(T_{\text{MRR}}\) increase was largely reflected in \(T_{\text{on}}\) increase. Note the difference in the y-axis scale between \((a)\) and \((b)\). Plotting the decrement of normalized MRR of AH- (open circles) and S-spikes (filled circles) evoked by the current injection into the AH \((c)\) and soma \((d)\) against the increment of \(T_{\text{MRR}}\). Note that the decrease in MRR per unit increase in \(T_{\text{MRR}}\) was invariably larger in AH-spikes than in S-spikes irrespective of the current injection sites.
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B: S-spikes evoked by current pulse injection into the soma before (black traces, a) and after application of 10 nM TTX (red traces, b) in the presence of 4-AP. Note the ramp-like depolarization preceding the S-spike and the long delay in triggering S-spikes (a). Horizontal interrupted lines in (a) and (b) indicate the peak level of the S-spikes with the minimal $T_{MRR}$ obtained before TTX application. The traces indicated with arrows in (a) and (b) were superimposed in inset (b) to show an increase in the threshold for evoking S-spikes with the same delay by TTX (inset, b). Also note the marked attenuation of the ramp-like depolarization by TTX. Plotting of $T_{MRR}$ against v on a linear (c) and a logarithmic scale (d), before (black circles) and after the application of TTX (red circles) in the presence of 0.2 mM 4-AP. Voltage and time calibrations in (a) also apply in (b).
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denoted with *3. In order to minimize artifacts included in the responses to stimulation of the stem axon, a response to a subthreshold stimulation of the stem axon was subtracted from all the responses shown in (b). C: Superimposed sample traces of simultaneously recorded AH-spikes (upper panels, a and b) and S-spikes (lower panels, a and b), obtained more than 360 sec after the application of 50 nM TTX. AH- and S-spikes (a) were evoked by injection of the stronger current pulse into AH, while the stimulation of the stem axon with 1.25 times the threshold intensity no longer evoked AH- and S-spikes (b). Horizontal arrows in (a) indicate the same potential levels as shown in (Ba). A response to a subthreshold stimulation of the stem axon was subtracted from all the responses shown in (b). Traces labeled with *1 to *7 were obtained sequentially in response to sequential stimulation (S1-S3) applied every 10 sec. D: Sample traces of the temporal derivatives of the simultaneously recorded AH- (a) and S-spikes (b) evoked by current pulse injection into the soma. Note that the timing of MRR of the AH-spike (open arrowhead) preceded that of the S-spike (filled arrowhead) evoked in response to soma depolarization. E: Plotting of the peak amplitude (a) and MRR (b) normalized to their control, and of the $T_{MRR}$ (c), of AH- and S-spikes (open and filled circles, respectively), against the time during perfusion of 50 nM TTX. X marks on the x-axis denote the failures of the axonal spike. In spite of the continuous decrease in the normalized amplitudes (a) and in the normalized MRRs (b), before and after blockade of the axonal spike, $T_{MRR}$ increased sharply just before the blockade of the axonal spike (c).
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FIG. 5. Differential sensitivity of S- and AH-spike to QX-314. A: Superimposed sample traces of AH- and S-spike (left and right panels, respectively, in a and b). AH- and S-spikes were simultaneously recorded by using two patch pipettes containing normal and 0.5 mM QX-314 internal solution, respectively. AH- and S-spikes (a) and those (b) evoked in response to injection of current pulses into the AH and soma, respectively. Voltage and time calibrations in (a) also apply in all other panels. B: Plotting of the spike amplitude (a) and MRR (b) normalized to the controls of the AH-spikes (open circles) and S-spikes (filled circles), partly shown in (A), against the time after rupture of the patch membrane of the QX-314-containing soma pipette. Both the spike amplitude and the MRR decreased more sharply in AH-spikes than in S-spikes, in spite of decreasing concentration gradient of QX-314 from the soma to AH (p < 0.001, ANOVA). C: The mean ± SD values of the normalized amplitude (a) and MRR (b) of the AH- (open circles) and S-spikes (filled circles), obtained 20-25 min after the establishment of dual whole-cell patch-clamp in 4 MTN neurons. The control was obtained 5-7 min after dual whole-cell recording. *: p < 0.05 (ANOVA). D: Plotting the increments of $T_{MRR}$ (circles), $T_{on}$ (triangles), and $T_r$ (diamonds) value of the AH-spikes evoked by current injection into the AH (a), and those of the S-spikes evoked by current injection into the soma (b), against the decrement of normalized MRR of respective spike. Note that $T_{MRR}$ increase was largely reflected in $T_{on}$ increase. Note the difference in the y-axis scale between (a) and (b). Plotting the decrement of normalized MRR of AH- (open circles) and S-spikes (filled circles) evoked by the current injection into the AH (c) and soma (d) against the increment of $T_{MRR}$. Note that the decrease in MRR per unit increase in $T_{MRR}$ was invariably larger in AH-spikes than in S-spikes irrespective of the current injection sites.