Spatial gradient in TTX sensitivity of axons at the crayfish opener neuromuscular junction

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Lin JW. Spatial gradient in TTX sensitivity of axons at the crayfish opener neuromuscular junction. J Neurophysiol 109: 162–170, 2013. First published October 10, 2012; doi:10.1152/jn.00463.2012.—At the crayfish opener neuromuscular junction, axons branch repeatedly before synapsing onto muscle fibers as varicosities. Excitability of these axons was examined with two-electrode current clamp before and after partial block of Na⁺ channels with 1 nM tetrodotoxin. 4-Aminopyridine (200 μM) was added to homogenize nonuniformity in K⁺ channel density. The impact of tetrodotoxin was evaluated in terms of action potential (AP) amplitude, rate of rise, and threshold. All three parameters were more severely affected at the secondary than the primary branching point (BP). Both BPs fired continuously during 1-s current steps before tetrodotoxin. After tetrodotoxin, the secondary BP fired only in brief bursts, whereas the primary BP still fired continuously. Despite this diminished excitability at the secondary BP, no failure in orthodromic AP conduction was observed. AP waveform at terminals (APf) was examined with voltage indicators. For orthodromic APs, reduction in AP amplitude and prolongation of AP rise time in tetrodotoxin were more pronounced in terminals than at the secondary BP. For APs initiated at the secondary BP, APf sometimes failed to show a spikelike waveform in tetrodotoxin. This degraded APf was not due to averaging variable AP invasion into terminals, because the variance of APf traces did not increase in tetrodotoxin. Tetrodotoxin applied in the absence of 4-aminopyridine showed an impact on the distal axon similar but less distinct than that recorded with 4-aminopyridine. In conclusion, the distal axon is more sensitive to tetrodotoxin than the proximal axon, such that AP waveform degrades as it propagates toward terminals in tetrodotoxin.

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Important in shaping the duration of AP at release sites (Leao et al. 2005). The kinetics and density of Na⁺ channels in the axonal initial segment, terminal boutons, and axonal blebs have been compared in hippocampal mossy fiber axons. The kinetic properties unique to each location were shown to be appropriate for the corresponding role of Na⁺ channels in AP initiation or propagation (Engel and Jonas 2005; Schmidt-Hieber and Bischofberger 2010).

One important element of axonal function that has been difficult to explore in detail is the spatial distribution of voltage-gated channels along a branching axon. Most axons in the mammalian CNS branch repeatedly as they approach their synaptic targets. Very little is known about the distribution of ion channels in such structures. Differential AP conduction failure at branching points (BPs) has been observed at the crustacean neuromuscular junction (Grossman et al. 1979a, 1979b; Parnas and Segev 1979; Smith 1980). This differential conduction failure was attributed to the accumulation of extracellular K⁺ during high-frequency firing. However, modeling studies of these results assumed a spatially uniform density of voltage-gated channels along the axon (Parnas and Segev 1979), whereas studies in other systems suggest that Na⁺ channel density may vary between different axonal compartments. Extracellular recordings from vertebrate neuromuscular junction (Brigant and Mallart 1982; Lindgren and Moore 1989) and intracellular recordings from crayfish sensory afferents (Cattaert and El Manira 1999; Cattaert et al. 2001) have indicated that some axons display reduced Na⁺ channel density in the terminal region. At the neuromuscular junction, it was suggested that a gradual shortening of the internodal length, as a motor axon approached muscle fibers, would ensure a sufficiently large forward charging current such that the presence of Na⁺ channels in terminals would not be essential (Lindgren and Moore 1989). In the case of the crayfish sensory afferent, the reduced Na⁺ channel density near the nerve ending was suggested to be essential for effective presynaptic inhibition mediated by GABA_A receptors (Cattaert et al. 2001).

To further investigate spatial variation in axonal excitability, it is necessary to be able to manipulate membrane potential (V_m) locally. Crayfish axons at the opener neuromuscular junction are morphologically similar to typical branching axons in the mammalian CNS (Florey and Cahill 1982) and are accessible to multiple electrode penetrations. As a result, they can serve as a model system for the study of membrane excitability in axons. In this report, two-electrode current clamp (TECC) conducted locally was used to explore regional variation in membrane excitability after Na⁺ channels were partially blocked by low concentrations of TTX.
METHODS

Preparation and recording. Crayfish, Procambarus clarkii, were purchased from Atchafalaya Biological Supplies (Raceland, LA). Small animals, 4–6 cm head to tail, were maintained in tap water at room temperature (22°C). All experiments were performed at 22°C. The first walking leg was removed by autotomy and fixed with crazy glue, dactylopodite side down, to a 15-mm petri dish. The opener axon-muscle preparation was dissected in saline. To ensure complete drug access to axons, the upper half of the shell of the carpopodite was removed such that the entire length of the axons was exposed to perfusing saline. Only the inhibitory axon was used in this study.

Physiological saline contained (in mM) 195 NaCl, 5.4 KCl, 13.5 CaCl₂, 2.6 MgCl₂, and 10 HEPES, titrated to pH 7.4 with NaOH. The glue, dactylopodite side down, to a 15-mm petri dish. The opener axon-muscle preparation was dissected in saline. To ensure complete drug access to axons, the upper half of the shell of the carpopodite was removed such that the entire length of the axons was exposed to perfusing saline. Only the inhibitory axon was used in this study.

Normalization of fluorescence transients was calculated as

\[ F(t) = \frac{F_{rest}}{F_{rest} \times 100} \]

where \( F_{rest} \) represents the fluorescence intensity of stained varicosities in the absence of activity. Background fluorescence in unstained regions was not subtracted. To objectively measure parameters characterizing AP waveform from fluorescence transients (\( A_P \)), a Gaussian function was used to fit \( A_P \). The curve fit started at \( -1.5 \) ms before the AP peak and extended to the falling phase of the AP. (See blue traces in Fig. 4, A2 and B2, for an example.) Since APs were asymmetric in shape, the curve fit spanned the entire rising phase but only part of the falling phase. The height of the Gaussian fit was taken as \( A_P \) amplitude, and the 10–90% rise time of the best fit was taken as \( A_P \) rise time.

RESULTS

A previous study using local TECC showed that AP threshold (\( V_{TH} \)) was higher at the secondary (2°) BP than at the primary (1°) BP (Lin 2012). This was attributed in part to the presence of a subthreshold K⁺ channel localized at the 2° BP. However, a small difference in \( V_{TH} \) was still present after subthreshold conductance was blocked by 200 \( \mu \)M 4-AP. To examine the possibility that nonuniformity in Na⁺ channel density may also contribute to this spatial variation in excitability, the impact of partially blocking Na⁺ channels on AP initiation was investigated by applying 1 mM TTX in the presence of 200 \( \mu \)M 4-AP.

Figure 1 illustrates TECC recordings obtained simultaneously from the 1° and 2° BPs. The separation of the two recording sites was ~1,000 \( \mu \)M. A threshold current (\( I_{TH} \)) of 15 nA initiated four APs at the 1° BP (Fig. 1A1, black). These APs in turn propagated to the 2° BP (Fig. 1A1, red). The APs initiated at the 1° BP exhibited a slightly earlier onset than those recorded at the 2° BP (Fig. 1A1, inset), suggesting orthodromic conduction. An \( I_{TH} \) of 9 nA initiated one AP at the 2° BP (Fig. 1B1, red), which propagated antidromically to the 1° BP (Fig. 1B1, black). Figure 1B1, inset, shows a longer conduction delay than that in Fig. 1A1, inset, suggesting that antidromic propagation velocity is slower than orthodromic. Introduction of 1 mM TTX did not alter \( I_{TH} \) at the 1° BP, but the number of APs initiated was reduced from four to two (Fig. 1A2). \( I_{TH} \) at the 2° BP rose from 9 to 10 nA in TTX. AP recorded at the 2° BP exhibited a visibly slower rising phase than that recorded in control saline (Fig. 1, B1 and B2, insets), although this AP appeared to be minimally altered by the time it reached the 1° BP (Fig. 1B2, inset, gray). The arrow in Fig. 1B2 identifies a subthreshold Na⁺ spike at the 2° BP. A detailed comparison of APs at their initiation sites, before and after exposure to TTX, is shown in Fig. 1, A3 and B3. When AP amplitude was measured from resting \( V_m \), i.e., before the onset of current steps, to the peak of the first AP initiated by \( I_{TH} \), AP at the 1° BP was reduced from 100 to 94 mV (Fig. 1A3, inset). The reduction was smaller than that measured at the 2° BP, which dropped from 101 to 85 mV (Fig. 1B3, inset).

Phase plots of APs recorded at current injection sites were also compared before and after TTX (Fig. 1, A4 and B4, and insets). At the 1° BP, the phase plot in TTX showed a reduction in the maximal rate of rise (Fig. 1A4). In addition, the initial rising phase of the phase plot showed a gradual ascent both before and after TTX (Fig. 1A4, inset), indicative of AP initiation at the current injection site. At the 2° BP, the reduction in the maximal rate of rise in TTX was more pronounced than that at the 1° BP (Fig. 1B4). The initial rising phase, although steeper than that at the 1° BP in control saline, still exhibited a take-off distinctly slower than the “kinks” typically observed for remotely activated AP (Fig. 1B4, inset) (Yu et al. 2008). Also noticeable in Fig. 1, A4 and B4, insets is the relatively large rise in AP threshold at the 2° BP in TTX, defined by the horizontal line at 10 V/s.

In six to nine preparations, AP amplitude reduction—as measured from the first AP fired by \( I_{TH} \)—in the presence of 1
nM TTX was 7.6 ± 2.0 mV (n = 6) at the 1° BP and 19.8 ± 2.8 mV (n = 9) at the 2° BP (P < 0.025). The maximal rate of rise of APs initiated at the 1° BP was reduced to 67.4 ± 3.2% (n = 6) of control levels in TTX, which was significantly less of a reduction than the 41.1 ± 4.1% (n = 9) measured at the 2° BP (P < 0.01). The reduction in orthodromically conducting AP initiated by a suction electrode placed ~3 mm proximal to the 1° BP, averaged 8.2 ± 1.0 mV (n = 5) at the 2° BP, which is statistically indistinguishable from that activated at the 1° BP and propagated to the 2° BP (10.1 ± 2.6 mV; n = 6). The rise in AP threshold induced by TTX was 3.8 ± 0.6 and 7.2 ± 0.5 mV (n = 5) at the 1° and 2° BPs (P < 0.05), respectively. Thus the analyses of parameters related to AP waveform and initiation suggest a preferential impact of 1 nM TTX on the 2° BP.

Since the main function of an axon is to propagate AP from soma to synaptic terminals, the reliability of AP conduction in an orthodromic direction in TTX was examined at the 2° BP. AP conduction failure in this direction never occurred. This finding was true for both orthodromic AP trains evoked by a suction electrode at 100 Hz for 500 ms (n = 8) and AP trains (60–96 APs) initiated by 1-s current steps injected at the 1° BP and recorded at the 2° BP (n = 6). Thus, although TTX at 1 nM clearly reduced excitability in the distal axon, the blocker did not cause conduction failure.

In addition to the larger reduction of AP amplitude in TTX, the rise of APs initiated at the 1° BP was reduced to 67.4 (9 nA) delivered at the 1° BP evoked 4 action potentials (APs) recorded at the 1° (black) and 2° (red) BPs. The schematic drawing illustrates recording configuration. Inset on right shows the first APs on an expanded timescale. A2: in the presence of 1 nM TTX, the same ITH evoked only 2 APs. Traces in inset show the first APs recorded at the 2° BPs in detail. The key to recording conditions and locations is applicable to all panels. A3: superimposed APs evoked and recorded at the 1° BP before (black) and after (gray) TTX. APs in inset were aligned with respect to their rising phases. A4: phase plot of APs shown in A3. Inset shows the initial rise of the phase plot in detail. Horizontal line at 10 V/s identifies AP threshold. B1: AP initiated at the 2° BP by ITH (9 nA). This recording was obtained from the soma to synaptic terminals, the reliability of AP conduction in this direction never occurred. This finding was true for both orthodromic AP trains evoked by a suction electrode placed ~3 mm proximal to the 1° BP, averaged 8.2 ± 1.0 mV (n = 5) at the 2° BP, which is statistically indistinguishable from that activated at the 1° BP and propagated to the 2° BP (10.1 ± 2.6 mV; n = 6). The rise in AP threshold induced by TTX was 3.8 ± 0.6 and 7.2 ± 0.5 mV (n = 5) at the 1° and 2° BPs (P < 0.05), respectively. Thus the analyses of parameters related to AP waveform and initiation suggest a preferential impact of 1 nM TTX on the 2° BP.

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the 2° BP fired only in brief bursts at the beginning of current steps. If AP initiation is more sensitive to partial block of Na\(^+\) channels at the 2° BP than the 1° BP, the next question is whether there is further degradation in AP waveform in the still more distal presynaptic terminals. Fluorescence transients of voltage indicator JPV1114 were used to monitor activity at terminal varicosities. Figure 4 illustrates two representative experiments. In the first case, APs were initiated by orthodromic stimulation and recorded at the 2° BP with a microelectrode (AP\(_f\)) and at the terminal with the voltage indicator (AP\(_e\)). AP\(_f\) recorded in TTX (Fig. 4A, red) showed a slightly lower amplitude than that measured in control saline (Fig. 4A, black). Figure 4A2 illustrates averaged AP\(_f\) (n = 15) recorded from terminal varicosities downstream from where AP\(_e\) was recorded. The reduction in amplitude and prolongation of rise time in TTX were more pronounced in AP\(_f\) than AP\(_e\) traces. The blue lines in Fig. 4A2 represent Gaussian fits to AP\(_f\) for the estimates of amplitude and 10–90% rise time. To evaluate the possibility that the changed AP\(_f\) waveform in TTX was due to occasional conduction failure, or jittering in the AP arrival time, the variance of fluorescence transients was calculated from the same trials used to obtain averaged AP\(_f\) (Fig. 4A3). There was no change in the variance level during the period of AP\(_f\). Thus there is no evidence for unreliable AP invasion into terminal varicosities in TTX. It should be noted that traces in Fig. 4A were recorded in the presence of 4-AP. It is possible that the presence of 4-AP-sensitive low-threshold \(i_h\) channels under physiological conditions could render AP invasion into terminals variable. Figure 4B illustrates measurements made in the absence of 4-AP. Similar to results obtained in 4-AP, the reduction in AP\(_e\) amplitude was less severe than that of AP\(_f\) (Fig. 4, B1 and B2) in the presence of TTX. Comparison of AP\(_f\) waveform before and after TTX washout showed a distinct slowdown in the AP\(_f\) rising phase while the preparation was in TTX. More importantly, the variance trace, indicative of the reliability of AP invasion into terminals, remained flat before and after TTX washout (Fig. 4B3). The flat variance traces were consistently observed in 12 preparations. Therefore, despite a significant degradation of AP waveform in 1 nM TTX, orthodromic AP invasion into terminals appears not to fluctuate under physiological conditions.

In 12 preparations where AP\(_f\) and AP\(_e\) were recorded simultaneously, the relative reduction in AP amplitude was compared by plotting the percent reduction in AP\(_f\) against that of AP\(_e\) recorded at the 2° BP. The reduction in AP\(_f\) was significantly larger than the reduction in AP\(_e\) at the 2° BP (Fig. 4C), and all but one data point were below the identity line (Fig. 4C, dashed line). The open square with error bars in Fig. 4, C and D, represents averages and SE for all data points. TTX-induced prolongation in AP rise time was significantly greater in AP\(_f\) than in AP\(_e\), and all data points were above the identity line (Fig. 4D). To illustrate that a similar effect of TTX also occurred under physiological conditions, the summary plots in Fig. 4, C and D, include data collected in the presence and absence of 4-AP (data points measured in the presence of 4-AP are indicated by circles). Data compiled from experiments performed under both conditions mixed randomly, suggesting that the TTX-induced deterioration in AP\(_f\) occurs regardless of the presence of 4-AP.

The invasion into terminals by APs initiated at the 2° BP was more variable. Figure 5A1 illustrates AP\(_f\) evoked by \(I_{TH}\) injected at the far (Fig. 5A1, black) and near (Fig. 5A1, red) BPs in control saline. (Far and near are defined with respect to the terminals imaged, indicated by the dotted red circle in the schematic in Fig. 5A1.) The AP\(_f\) evoked by the near electrode \((I_{TH} = 8 \text{ nA})\) was characterized by an elevated DC level before AP\(_f\), which presumably resulted from passive spread of depolarization from the near injection site. Furthermore, the onset of AP\(_f\) initiated by the near electrode occurred earlier than its corresponding AP\(_e\), reflecting the fact that the distance between the near electrode and imaged terminals was shorter than that...
to the peak (Fig. 5A1, double-headed arrow). The averaged ratio was 96.8 ± 2.5% (n = 8) in control saline and 85.1 ± 3.7% in TTX (P < 0.05) (Fig. 5C). This suggests that, after Na\(^+\) channels are partially blocked, AP initiated at the 2\(^\circ\) BP may fail to support full-sized AP in nearby terminals, whereas orthodromically propagating AP can still invade and initiate well-defined AP waveforms in these terminals.

The impact of 1 nM TTX under physiological conditions, i.e., without 4-AP, was also examined with simultaneous TECC. Figure 6A1 shows a preparation in which 1 nM TTX reduced excitability at the 1\(^\circ\) BP by raising \(I_{TH}\) from 17 to 23 nA. Expansion of APs near the onset of the current step (Fig. 6A1, horizontal bar) showed a reduction of 3 mV in the amplitude of the first AP fired by \(I_{TH}\) (Fig. 6A2). A phase plot of the initial spikes showed that there was a reduction in the maximal rate of rise (Fig. 6A4) but no change in AP threshold (Fig. 6A4, inset). The current dependence of firing at the 1\(^\circ\) BP was reduced in TTX, but the rising trend remained clear (Fig. 6A3). The impact of TTX on the 2\(^\circ\) BP of the same preparation was also clear (Fig. 6, B1 and B2). Whereas the increase in \(I_{TH}\) was smaller, from 17 to 20 nA, than that observed at the 1\(^\circ\) BP, the reduction in the amplitude of the first AP was large (8 mV). A current step series evoked high-frequency bursts before addition of TTX (Lin 2012) but could only activate two APs over the same range of current steps (Fig. 6B3). The phase plot shows that there was a large reduction in the maximal rate of rise (Fig. 6B4) but no significant change in the threshold of the first AP after addition of TTX (Fig. 6B4, inset). In the same preparation, an orthodromic train of 51 APs at 100 Hz showed no conduction failure or jittering in conduction speed at the 2\(^\circ\) BP (data not shown).

In seven preparations in which the impact of 1 nM TTX on the 2\(^\circ\) BP was tested in the absence of 4-AP, two axons failed to fire AP in response to current steps of up to 40 nA, −2 × control \(I_{TH}\). In animals in which 1 nM TTX did not completely eliminate AP initiation at the 2\(^\circ\) BP, the differential effect of TTX on the two BPs was not as distinct as that observed in preparations treated with 4-AP (Figs. 1–5). Specifically, the amplitude of the maximal AP fired by \(I_{TH}\) was the only parameter showing a significant difference in sensitivity to TTX between BPs. The maximal AP amplitude was decreased by 3.3 ± 1.3 mV (n = 7) at the 1\(^\circ\) BP and by 7.2 ± 1.0 mV (n = 5) at the 2\(^\circ\) BP (P < 0.05). The impact of TTX on the amplitude of the first AP fired by \(I_{TH}\) on \(V_{TH}\) and on the maximal rate of rise at the two BPs was not statistically different. No orthodromic AP conduction failure was observed in the presence of TTX (n = 5). Thus, because of the large current needed to initiate AP in the presence of 4-AP-sensitive \(i_{K}\) in the distal axon, the differential impact of TTX on the two BPs was not statistically different. No orthodromic AP conduction failure was observed in the presence of 4-AP.

**DISCUSSION**

Results in this report show that the distal compartment of axons at the crayfish opener neuromuscular junction is more sensitive to 1 nM TTX than the proximal compartment. This differential sensitivity is reflected by a larger reduction in AP amplitude, a raised AP threshold, and an altered firing pattern at the more distal 2\(^\circ\) BP. For APs propagating in an orthodromic direction this spatial gradient in TTX sensitivity re-
resulted in no conduction failure, but the degradation of AP waveform in terminal varicosities was more pronounced than that recorded at the 2° BP. The degraded AP waveform was not a result of averaging fluctuating APs in terminals, given that no change in variance was observed in AP traces after addition of TTX. The working hypothesis for these results is that the distal axon and terminals may have a lower Na⁺ channel density than the proximal axon. The physiological significance of this apparent spatial gradient in Na⁺ channel distribution is discussed below.

Differential impact of TTX on the two BPs is less apparent in physiological saline than in 4-AP. One puzzling finding in this report is that the preferential impact of TTX on the distal axon was more apparent in the presence of 4-AP. Because of the strong dampening impact of 4-AP-sensitive subthreshold i_K localized in the distal axon (Lin 2012), one would expect that TTX, with the help of the i_K, would render the distal axon nonexcitable. This was indeed the case in two of the seven preparations tested, where 1 nM TTX prevented AP initiation at the 2° BP. However, in the remaining five animals, 1 nM TTX did not significantly lower the amplitude of the first AP fired by I_TH or the maximal dV/dt or raise V_TTH. The only parameter more severely affected by TTX at the 2° BP was the maximal AP amplitude fired by I_TH. One likely explanation would be that the presence of high-density 4-AP-sensitive i_K at the 2° BP caused I_TH in that region to be comparable in amplitude to that at the 1° BP, despite the fact that the actual input resistance at the 2° BP was about twice that at the 1° BP (Lin 2012). Since injected current can contribute to the shaping of AP waveforms, the relatively large I_TH at the 2° BP may render the impact of partially blocking i_Na on AP waveform less detectable in physiological saline.

Mechanisms underlying the nonuniformity in TTX sensitivity. Investigations of the correlation between i_Na and AP amplitude in pyramidal cell soma suggested that, because of the abundance of Na⁺ channels, AP amplitude was not affected until >50% of Na⁺ channels were blocked by TTX (Madeja 2000). Since there are no i_Na data for axons in the crayfish opener preparation, the fraction of Na⁺ channels blocked by 1 nM TTX is unknown. Two possibilities could explain the results reported here. The first would be that Na⁺ channels in the distal axon belong to an isoform different from those in the proximal axon, and may have a higher sensitivity to TTX. Investigation of this hypothesis would require voltage clamp, immunocytochemistry, and detailed pharmacological comparison of i_Na in the proximal and distal axon. The second possibility is that cray-fish axons express a single class of Na⁺ channel but with decreasing channel density as axon branches taper and become strings of varicosities. In this scenario, Na⁺ channel density in the distal axon would be sufficient to shape AP in control saline, but there would be no excess channels there. Since TTX at 1 nM would block a constant fraction of Na⁺ channels over the entire length of the axon, this would reduce the number of functional Na⁺ channels in the distal axon to below a critical level such that AP amplitude, threshold, and firing pattern would be clearly affected. The functional significance of this hypothesis is consistent with the recent finding that Na⁺ and K⁺ current kinetics are tuned to minimize ion flux for the generation of a given AP shape (Schmidt-Hieber and Bischofberger 2010). The minimal ion flux in turn reduces the metabolic load on thin axons and varicosities, where the large surface-to-volume ratio makes these compartments prone to activity-dependent changes in intracellular ionic concentrations. In this context, it would be functionally advantageous to
place a high density of Na\(^+\) channels in the large proximal axon in order to generate a sufficiently large forward charging current and to ensure a full-sized AP in all thin axons and terminals (Lindgren and Moore 1989). This hypothesis also implies that BP failure is unlikely to occur under physiological conditions, since AP in terminals would be mainly generated by forward charging current, with Na\(^+\) channels in distal axons playing only a facilitating role in shaping AP, by “touching up” the AP rising phase. In a different functional context, the proposed low-level Na\(^+\) channel density in the distal axon together with the high density of low-threshold K\(^+\) channels (Lin 2012) may contribute toward minimizing unintended local AP initiation resulting from depolarization generated by local injuries or by presynaptic ionotropic and metabotropic receptors.

Neuronal firing patterns are determined by complex interactions between inward and outward current (Bean 2007; Tateno et al. 2004). Given the limited information available on the kinetics of voltage-gated channels in crayfish axons, the effect of TTX on the firing pattern at the 2° BP can only be speculated upon. Nevertheless, similar changes in firing pattern have been reported elsewhere after Na\(^+\) channel availability was reduced. Expression of a mutant Na\(^+\) channel in sympathetic ganglion neurons resulted in depolarized resting \(V_m\) and changes in firing pattern similar to those reported here at the 2° BP after TTX (Rush et al. 2006). The change in firing pattern was attributed to reduced Na\(^+\) channel availability due to depolarization-induced inactivation. In mammalian central neurons, blocking a persistent component of Na\(^+\) current with riluzole resulted in changes in firing pattern similar to those seen here at the 2° BP (Theiss et al. 2007; Wu et al. 2005). Thus the TTX-mediated change in firing pattern at the 2° BP reported here is not without precedent and may simply reflect a common response to a large reduction in Na\(^+\) channel density.

**Impact of partially blocking Na\(^+\) channels on forward AP propagation.** Although a clear reduction in membrane excitability at the 2° BP was observed when the axon was probed by current steps, the percent reduction in AP amplitude propagated orthodromically was similar at the two BPs. In addition, there was a further degradation in AP waveform at terminal varicosities in TTX compared with AP\(_c\) recorded at the 2° BP. While the reduction in orthodromic AP amplitude was \(~10\%\) at the two BPs, the reduction was \(~25\%\) at terminal varicosities (Fig. 4C). Since the variance of fluorescence transients did not increase as APs arrived at terminals (Figs. 4 and 5), the possibility that the degraded AP\(_c\) waveform was due to jittery or fluctuating invasion in TTX can be ruled out. Rather, the reduced amplitude and prolonged rise time most likely reflect true changes in terminal AP waveform. According to this scenario, the terminal region would become almost passive in true changes in terminal AP waveform. According to this scenario, the terminal region would become almost passive in 1 nM TTX and the AP\(_c\) recorded in TTX would reflect filtered AP invading terminal regions. This interpretation is consistent with the failure in some preparations to observe discernible AP shape in terminals when AP was initiated at the nearby 2° BP.

In this case, not enough charging current would have been generated by the AP initiated locally and there would not have been enough functional Na\(^+\) channels in terminals to support a spike-shaped depolarization. Furthermore, the subthreshold de-
polarization preceding the AP was \( >30 \) mV at the 2° BP (Fig. 1B2) and significant at terminals (Fig. 5A2), such that a significant fraction of Na\(^+\) channels spared by TTX could be inactivated. These factors combined could explain the absence of spike shape in Fig. 5A2.

It should be noted that results in Fig. 5 showed that the degree of terminal invasion by an AP initiated at the 2° BP was variable among preparations in TTX. A more consistent pattern may emerge if morphological details of the relationship between the terminal varicosities imaged and the 2° BP where \( \text{AP}_p \) was initiated are known.

**AP amplitude in terminal varicosities.** A major limitation in the use of voltage indicators is the lack of calibration in areas where direct electrophysiological recordings are not possible. Recordings in Fig. 5 provide an estimate of AP amplitude in terminals. When current steps delivered to the 2° BP failed to initiate AP in terminals (Fig. 5A2), the peak level of depolarization there was \( \sim 80\% \) of \( \text{AP}_p \) initiated from the 1° BP. Assuming that subthreshold depolarization at the 2° BP reached approximately \( -40 \) mV (Fig. 1B4), starting from a resting \( V_m \) of \( -80 \) mV, then the amplitude of subthreshold depolarization in terminals could not be more than \( 40 \) mV after passive decay from the 2° BP. Averaged results in Fig. 5C indicate that \( \text{AP}_f \) invading from the 1° BP was about 1.13-fold higher than the peak level initiated from the 2° BP and should, therefore, be no more than \( 45 \) mV. The estimated \( 45 \) mV \( \text{AP}_f \) was recorded in TTX, and, according to Fig. 4C, this value should be \( \sim 75\% \) of control amplitude. Therefore, AP amplitude in terminals without TTX would be \( \sim 60 \) mV. Since this \( 60 \) mV amplitude is likely to be an overestimate, and is significantly smaller than the 80 mV \( \text{AP}_p \) recorded at the 2° BP in 200 \( \mu \text{M} \) 4-AP (Lin 2012), it is reasonable to conclude that there may already be significant attenuation of AP amplitude in terminals under physiological conditions. This conclusion offers a good explanation for the results of an earlier study of the crayfish opener neuromuscular junction, where it was suggested that most terminal varicosities are not excitable (Dudel 1983). In that study, transmitter release evoked by a macro-patch electrode positioned over terminal varicosities was found to be graded according to the current passed by the electrode, rather than showing an all-or-none response, and this graded release was not affected by TTX. Although this explanation for graded release and the absence of TTX sensitivity could be confounded by the presence of low-threshold, fast-activating \( K^+ \) channels in terminals (Lin 2012), it is also consistent with the idea that terminals have lower Na\(^+\) channel density and excitability than more proximal regions.

In conclusion, under physiological conditions a high Na\(^+\) channel density in the proximal axons of this preparation appears to endow it with a large safety margin to ensure reliable AP invasion into terminal varicosities and thus reliable synaptic transmission.

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**DISCLOSURES**

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**AUTHOR CONTRIBUTIONS**

Author contributions: J.-W.L. conception and design of research; J.-W.L. performed experiments; J.-W.L. analyzed data; J.-W.L. interpreted results of experiments; J.-W.L. prepared figures; J.-W.L. drafted manuscript; J.-W.L. edited and revised manuscript; J.-W.L. approved final version of manuscript.
REFERENCES

Dudel J. Graded or all-or-nothing release of transmitter quanta by local depolarizations of nerve terminals on crayfish muscle? Pflügers Arch 398: 155–164, 1983.
Madeja M. Do neurons have a reserve of sodium channels for the generation of action potentials? A study on acutely isolated CA1 neurons from the guinea-pig hippocampus. Eur J Neurosci 12: 1–7, 2000.