Na⁺ current in presynaptic terminals of the crayfish opener cannot
initiate action potentials.

By
Jen-Wei Lin
Department of Biology
Boston University

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Communicating author: Jen-Wei Lin (jenwel@bu.edu)

Address: 5 Cummington Mall
Boston, MA 02215

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Abstract

Action potential (AP) propagation in presynaptic axons of the crayfish opener neuromuscular junction (NMJ) was investigated by simultaneously recording from a terminal varicosity and a proximal branch. Although orthodromically conducting APs could be recorded in terminals with amplitudes up to 70 mV, depolarizing steps in terminals to -20 mV or higher failed to fire APs. Patch clamp recordings did detect $i_{Na}$ in most terminals. The $i_{Na}$ exhibited a high threshold and fast activation rate. Local perfusion of Na$^+$-free saline showed that terminal $i_{Na}$ contributed to AP waveform by slightly accelerating the rising phase and increasing the peak amplitude. These findings suggest that terminal $i_{Na}$ functions to "touch-up" but not to generate APs.
Presynaptic terminals are not universally excitable. Electrotonically compact terminals such as the calyx of Held (Leao et al. 2005) and some vertebrate NMJs exhibit low excitability (Brigant and Mallart 1982). The \( \text{i}_{\text{Na}} \) density in the calyx of Held was found to be low, and Na' influx at the heminode was assumed to provide the current for full-height APs at release sites (Leao et al. 2005). Although the vertebrate NMJ exhibits considerable variation in morphology across species and locations, a significant proportion of motor end plates are morphologically compact, with 2-4 orders of branching within a space of 20X60 \( \mu \text{m} \) (Kang et al. 2014). Compact NMJ terminals are either not excitable or have low excitability (Brigant and Mallart 1982; Krzemien et al. 2000). By contrast, the axons with varicosities of CA3 mossy fibers (MF), the posterior pituitary and fast spiking interneurons are thin and long, extending for 500-1000 \( \mu \text{m} \), and can fire APs (Geiger and Jonas 2000; Hu and Jonas 2014; Jackson and Zhang 1995). High excitability is essential for action potential to reach the distal ends of such axons. These studies suggest a general trend that, whereas terminals with low excitability need to be electrotonically compact to function, axons with long, thin arborizations are typically highly excitable so that AP can propagate to distal terminals.

Since terminals of the crayfish opener NMJ are organized in a "beads on a string" configuration and can extend for hundreds of micrometers (Florey and Cahill 1982), it is surprising that previous studies have hinted at low excitability for these terminals. Graded stimulations delivered by macropatch pipettes evoked all-or-none transmitter release in proximal varicosities but only graded release in distal ones (Dudel 1983). The latter behavior suggests the presence of non-spiking terminals. A recent study using a voltage indicator to monitor AP in terminals of the same preparation reached a similar conclusion (Lin 2013). In the present report, direct recordings from terminals were used to investigate the role of \( \text{i}_{\text{Na}} \) in terminals with low excitability.

Methods

Crayfish (Procambarus clarkii), 5 cm head to tail, of both sexes were used. Treating a dissected opener of the first walking leg with Protease XIV (1 mg/ml, Sigma-Aldrich) for 15 minutes "unsheathed" some glia cells surrounding varicosities. Terminals were identified by the fluorescence marker Alexa-568 injected before protease treatment. Patch clamp of terminals was performed on both excitors and inhibitors. Only inhibitors were used for simultaneous axon and terminal current clamp studies because AP firing above 20Hz in the...
excitor triggered muscle contraction and would tear a preparation weakened by the enzyme.

The preparation was maintained in crayfish saline containing (in mM): 195 NaCl, 5.4 KCl, 13.5 CaCl₂, 2.6 MgCl₂, and 10 HEPES (pH=7.4). Sharp electrodes (1 M K-methansulfonate; ~40 MΩ) were used in two electrode current clamp (CC) at the primary (1°) branching point (BP) (IE-210, Warner Instrument). Patch clamp was performed with Axopatch 200B. Patch pipette (~10 MΩ) capacitance was minimized by wrapping parafilm around the pipette shank, to ~3 mm from the electrode tip. A K⁺ based solution used for current clamp contained (in mM): 180 K-gluconate; 12 KCl; 0.2 CaCl₂; 1 NaEGTA; 10 NaHEPES (pH=7.4); 5 MgATP; 0.3 KGTP; 20 Tris-phosphocreatine. K-gluconate was replaced by Cs-methansulfonate for \( i_{Na} \) recording. Liquid junction potential was not corrected. \( i_{Na} \), 10 kHz filtered an 15 μs sampled, was activated by 20 ms steps and conditioned by a 100 ms prepulse to -100 mV. Capacitive and leak currents were subtracted with the P/n procedure. Calcium current was not detectable in outside-out patches.

When Axopatch 200B was used in CC mode (I-Clamp Fast), electrode capacitance was compensated by complete cancelation of the capacitive current after gigaohm seals had formed. The bridge balance was achieved by dialing the correct electrode resistance first and, after switching to CC mode, setting series resistance compensation to 100%.

Since using the patch clamp amplifier in CC mode has been reported to distort AP shapes under certain conditions (Magistretti et al. 1998), CC recordings were also performed with a voltage follower type amplifier (Warner IE-210), with a custom-modified headstage for low current injection. The distributions of AP amplitude, duration and after-hyperpolarization recorded with Warner IE-210 (n=8) overlapped with those recorded with Axopatch 200B (n=15). (See scatter plots on AP coupling ratio in Fig.1D for example.)

The series resistance compensation was switched off in voltage clamp mode. Errors resulting from series resistance should be minor because: (1) \( i_{Na} \) recorded in outside-out configuration was small, <100 pA, and (2) only experiments with an access resistance of less than 40 MΩ were used for analysis. All averaged data values are expressed as mean±sem (standard error of means).

Local perfusion of Na⁺-free saline was delivered by pressure ejection through a micropipette, tip opening≈15 μm. The NaCl in control saline was replaced by choline chloride in Na⁺-free saline. Outflow of Na⁺-free saline was confirmed by the appearance of fluorescence (1 μM Alexa-568) at the tip of the perfusion pipette. \( i_{Na} \) recorded from a varicosity was eliminated after local perfusion was switched on (Fig.3A and C). Perfusion was assumed to be localized because: (i) the perfusion pipette tip was within 20 μm of patched terminals, (ii) local perfusion outflow (~0.5 μl/min) was ~2000 times
slower than the bath perfusion rate (~1 ml/min). The localized nature of perfusion was supported by the observation that APs recorded at the 1° BP, ~50-200 μm from perfused terminals, were never affected by perfusion.

**Results**

An AP train initiated at the 1° BP (Fig.1A1 blue) evoked corresponding transients in the on-cell patch of a terminal (Fig.1A1 red). The shape of on-cell transients (Fig.1A2) represents a combination of inversion and the time derivative of intracellular counterparts (Sasaki et al. 2012). Whole cell access to the terminal, with a holding potential of -80 mV, gave rise to a holding current of -250 pA. The capacitive current evoked by a -10 mV command decayed with two exponential components, with time constants (τ) of 0.06 and 0.8 ms in the example shown in Fig.1B2. Voltage steps uncovered a mixture of early $i_{Na}$ and late $i_K$ (Fig.1B1) with the K+ based pipette solution.

Capacitive transients compiled from 18 terminals gave rise to averaged fast and slow τ of 0.13±0.01 and 1.33±0.16 ms, respectively. Assuming that the fast component was attributable to the capacitance of the patched varicosities (Geiger and Jonas 2000), and a specific membrane capacitance of 1μF/cm², an averaged varicosity corresponds to a sphere with a radius of 3.0±0.18 μm. This dimension is similar to the size of opener terminals reconstructed from serial electron microscopic sections (Cooper et al. 1995). The input resistance of crayfish varicosities, measured from the DC current evoked by a -10 mV step, was 168.5±46.8 MΩ (n=18) and significantly lower than that of MF boutons (>1 GΩ) of comparable size (~2 μm) (Engel and Jonas 2005; Geiger and Jonas 2000).

Under current clamp, resting $V_m$ at the terminal was -58.4±2.1 mV, while that at the 1° BP was -74.9±1.5 mV (n=15). Subthreshold depolarization at the 1° BP (Fig.1C1 lower blue dash) resulted in a similar potential at the terminal (upper red dash). (See C2 upper panel for recording configuration.) APs evoked at the 1° BP spread to the terminal (Fig.1C1 thin traces), but their amplitude was reduced to half (Fig.1C2 lower panel). The reduction in AP amplitude in the terminal despite strong DC coupling suggests that these terminals may not be active. Supporting this possibility, depolarizing the terminal up to -35 mV failed to fire any APs (Fig.1C3 red thick); subthreshold depolarization initiated at the 1° BP delivered simultaneously with a 200 pA step in the terminal further depolarized $V_m$ to -25 mV and still failed to fire AP (Fig.1C3 dashed red). APs fired at the 1° BP spread to the depolarized terminal with slightly more attenuation than that
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observed without terminal depolarization (Fig.1C2 and C4 lower panels).

Electrotonic coupling of passive signals between the $1^\circ$ BP and terminals was asymmetrical. While a 30 mV depolarization in the terminal (Fig.1C3 red thick) failed to induce any change at the $1^\circ$ BP (Fig.1C3 blue thick), an 18 mV hyperpolarization at the $1^\circ$ BP (Fig.1C4 upper blue) caused a 17 mV potential in the terminal (Fig.1C4 upper red). When the orthodromic coupling ratios of action potentials were plotted against those of passive signals initiated with a -20 nA and 20 ms step at the $1^\circ$ BP, the majority of data points fell below the identity line (Fig.1D). Open and filled circles represent data recorded from Warner IE-210 and Axopatch 200B, respectively. This distribution pattern is consistent with the expectation that passive cables attenuate fast transients more readily than slow signals.

In 54 terminals studied under current clamp, none fired an AP over a depolarization range of -35 to +5 mV. In 15 preparations where simultaneous BP and terminal recordings were made, AP amplitude at the two locations was 73.2±2.8 and 41.5±5.3 mV, respectively. In 19 of these terminals, voltage clamp was also performed. All 19 terminals exhibited $i_K$ and 16 of them showed $i_{Na}$. However, the whole-cell $i_{Na}$ potentially could have come from connecting axons, neighboring varicosities or nearby tertiary branches. Outside-out patch was used to determine whether $i_{Na}$ was present in the patched varicosities.

The whole-cell $i_{Na}$ in Figure 2A exhibited unclamped spikes when voltage steps were above +10 mV. The poorly space clamped $i_{Na}$ were more frequently observed when the Cs$^+$-based pipette solution was in use. The inset in Fig.2A2 shows capacitive current recorded before (black) and after (grey) withdrawal of the patch pipette. Holding current was reduced from 263 to 8 pA. The capacitive transient evoked by a 10 mV step dropped from 246 to 42 pA and exhibited a single exponential decay. In twelve terminals with detectable whole-cell $i_{Na}$, three showed no $i_{Na}$ after pipette withdrawal. In the remaining nine endings, $i_{Na}$ was reduced to 28.7±9.6% of the whole terminal level, from 887±387 to 85±31 pA. Thus, a significant fraction of terminal varicosities have $i_{Na}$ channels. Fig.2A2 illustrates $i_{Na}$ recorded after pipette withdrawal from the terminal used in A1. $i_{Na}$ recorded from the excised patch exhibited rapid activation and gave rise to an I-V curve with an extrapolated reversal potential (+75 mV) consistent with $E_{Na}$ (Fig.2A3 open circle). Also included in Fig.2A3 are data from 8 additional preparations (in different symbols) and the averaged results (filled circles, n=9). $i_{Na}$ in terminals shows a threshold at -40 mV and peaks around 0 mV. The G-V curve calculated from the same data shows 50% activation at -11 mV (Fig.2C).
Steady state inactivation was investigated with 100 ms conditioning pulses and tested with a step to 0 mV. Complete inactivation of $i_{Na}$ occurred when the conditioning step was -30 mV (Fig.2B1). Data compiled from the same preparations used in Fig.2A3 show that 50% inactivation occurred at -51 mV (B2), close to the averaged resting $V_m$. The voltage dependence of both activation and inactivation is right-shifted by ~10 mV when compared to that recorded from the medial giant axon of the crayfish ventral nerve cord (Shrager 1974).

Although the activation time constant has not been curve-fitted for Hodgkin-Huxley modeling, the $i_{Na}$ had a time-to-peak rise time of 0.21±0.03 ms at 0 mV (n=9), comparable to rise times for $i_{Na}$ found in axons or terminals capable of firing AP in the mammalian CNS (Engel and Jonas 2005; Hu and Jonas 2014). The rapid kinetics of terminal $i_{Na}$ suggests that it should contribute to AP waveform. To evaluate this contribution, the charging current associated with an invading AP train was compared to the amplitude of $i_{Na}$ in the same terminal. A charging current of ~2 nA was recorded when the terminal was held at -80 mV (Fig.3B). A voltage step series in the same terminal evoked a mixture of $i_{Na}$ and $i_K$, with a maximal $i_{Na}$ of 80 pA (Fig.3A). Since amplitude measurements of both whole-cell $i_{Na}$ and charging current are of limited accuracy—due to issues related to space clamp, lack of $i_K$ block and uncorrected series resistance for large charging currents—these parameters were not statistically compared. Nevertheless, the large difference between the two currents, and the failure of large terminal depolarization to induce APs, suggest that APs in terminals are mainly created by charging current, and that local $i_{Na}$ may shape AP waveform but cannot generate an AP. This hypothesis was further tested by comparing AP waveform before and after removal of local Na'. Onset of local Na'-free saline perfusion immediately eliminated $i_{Na}$ recorded from a varicosity (Fig.3A and C). (The concomitant reduction of outward current was in part due to removal of Na' activated K' current.) Local perfusion had no impact on APs initiated and recorded at the 1° BP (Fig.3D axon), but slightly slowed the rising phase and reduced the peak amplitude of APs in the terminal (Fig.3D term).

Similar effects were found in 6 of 8 terminals tested. In the remaining two terminals, Na'-free saline had no effect on terminal APs. Since the precise area perfused by Na' saline could not be defined owing to the opener shell disrupting laminar flow of saline, effects shown here could be due to elimination of $i_{Na}$ from more than one varicosities. Nevertheless, these observations are consistent with the hypothesis that local $i_{Na}$ contributes to the shaping of invading AP in opener terminals.

Discussion
This report shows that APs in varicosities of the opener axon were mainly generated by charging current from proximal branches, and that terminals cannot generate APs. However, a small $i_{Na}$ with a high threshold and fast activation rate was present in most terminals. The fast kinetics and small amplitude of $i_{Na}$ suggested an auxiliary role, supported by the observation that local perfusion of Na⁺-free saline slightly decelerated AP rising phase and reduced AP amplitude. This report demonstrates a subtle and "quantitative" role for $i_{Na}$ in presynaptic terminals with low excitability.

Motor nerve endings could potentially experience large fluctuations in resting $V_m$. The large mass of skeletal muscle means that vigorous contraction can raise extracellular K⁺ concentration locally and depolarize terminals. Furthermore, motor nerve terminals have a variety of presynaptic receptors that can shift resting $V_m$ (Beaumont and Zucker 2000; Darabid et al. 2014; Wojtowicz and Atwood 1985). Presynaptic terminals therefore need to operate over a range of $V_m$ without backfiring. Low $i_{Na}$ magnitude reported here could be a contributing factor toward minimizing backfiring. Another potentially important parameter is the large leak current and low input resistance recorded at resting $V_m$. The input resistance reported here was ~50% of that in MF boutons, after correcting for size differences (Engel and Jonas 2005). Finally, low $i_{Na}$ could minimize the metabolic load on terminals (Alle et al. 2009). These considerations may also apply to the mammalian CNS, where local ionic and neurotransmitter concentrations can fluctuate due to restricted extracellular space.

Studies derived from mammalian CNS axons with strings of varicosities have consistently shown that they have a high Na⁺ channel density and can fire APs (Engel and Jonas 2005; Hu and Jonas 2014; Jackson and Zhang 1995). The low excitability of the crayfish opener axon, despite its long and "beads on a string" morphology, is surprising and raises the question of how APs may reach the distal varicosities. The unique adaptation of the crayfish opener axons may lie in the large diameter ratio between proximal (~30 µm) to distal (<1 µm) branches (Florey and Cahill 1982). Small $i_{Na}$ in terminals could play a boosting role in aiding AP spreading. Although a diameter ratio comparable to that of crayfish opener axons is not present in the mammalian nervous systems, a gradual reduction in internodal length have been suggested as a mechanism for accumulating charging current to depolarize motor end plates (Lindgren and Moore 1989; Quick et al. 1979). Morphological analyses of dorsal horn interneurons have reported thin, myelinated axons in close proximity to unmyelinated daughter branches with varicosities (Szucs et al. 2013). The myelinated compartment could potentially play a role in providing charging current for distal varicosities with low excitability. Thus,
terminal varicosities with low excitability could function, with advantages outlined above, in the mammalian CNS.

The data presented here suggest a dominant role for proximal branches of crayfish opener axons in generating terminal APs. However, the interactions between proximal and distal compartments need not be unidirectional because the large number of terminals could collectively influence the $V_m$ of proximal branches. The possibility of bidirectional interaction between axonal compartments and the roles $i_{Na}$ may play in these interactions remain to be further explored.
References


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Figure 1: Presynaptic terminals of the crayfish opener NMJ are not excitable. A1: On-cell patch from a terminal detects biphasic transients (red) corresponding to APs evoked by a 14 nA step at the 1° BP (blue). A2: Averages of on-cell transients (red) and APs (blue) from the traces in A1. B1: Whole cell current of the same terminal exhibits a mixture of $i_{Na}$ and $i_K$, activated by voltage steps starting at -50 mV with 20 mV increments. B2: Capacitive and leak currents activated by a -10 mV step. The black line represents a double exponential fit to the initial 5 ms of the transient. C1: Simultaneous current clamp recordings from the 1° BP (blue) and the terminal (red). Current steps delivered at the 1° BP were 0, 10 and 12 nA. C2: Upper panel: Recording configuration. Lower panel: APs marked by the rectangle in C1, aligned from their baselines. C3: A 200 pA step at the terminal depolarizes $V_m$ to -35 mV but does not evoke AP (upper thick red) and does not spread to the 1° BP (lower thick blue). Current steps injected at the 1° BP, simultaneously with the 200 pA step in the terminal, evoke subthreshold depolarization (thin dashes) and an AP train (solid thin traces). C4: Upper panel: Hyperpolarization generated at 1° BP (blue) spreads to the terminal (red) with little attenuation. Lower panel: Aligned APs corresponding to those identified by the rectangle in C3. D: AP coupling ratio (CR) plotted against passive CR from 23 preparations. CR was calculated as the ratio of amplitudes recorded at a patched terminal to that at the 1° BP. Filled circles were recorded from Axopatch 200B (n=15) and open circles (n=8) from Warner IE-210. Except for the upper panel of C4, all voltage traces share the vertical scale in A1. A1, C1 and C3 share the same time scale. Lower panels in C2 and C4 share the same time scale. All traces were obtained from the same terminal with K+ based pipette solution and with Axopatch 200B.

Figure 2: Kinetics of $i_{Na}$ recorded from opener terminals. A1: Whole-cell currents of a terminal activated by voltage steps from -80 to +40 mV with 10 mV increment. A2: $i_{Na}$ activated by the indicated voltage steps of an outside-out patch excised from the terminal used in A1. Inset: Capacitive and leak currents, evoked by a 10 mV step, recorded before (black) and after (grey) patch excision. A3: Normalized I-V curve recorded from nine preparations, in different symbols. Averaged data with error bars are in filled circles. The grey curve was calculated from the Boltzmann fit of the activation curve (C) and $E_{Na} = 75$ mV. B1: $i_{Na}$ inactivation mediated by 100 ms conditioning steps and tested with a 20 ms step to 0 mV. B2: Normalized inactivation responses from nine preparations, in different symbols. Averages are in filled circles. Averaged resting $V_m$ at terminals is indicated. C: Normalized activation curve calculated from the I-V curve shown in A3 with Boltzmann fit (grey). All traces and data points in this figure were obtained with Cs+ based pipette solution.
Figure 3: Contribution of terminal $i_{Na}$ to AP waveform. A: Whole cell $i_{Na}$ and $i_K$ recorded from a terminal. B: Voltage clamp recording of invading, charging current (upper trace) evoked by an AP train (lower trace) initiated at the 1° BP. C: Perfusion of Na'-free saline eliminates the $i_{Na}$ and $i_{K(\text{Na})}$ shown in A. D: Local perfusion of Na'-free saline does not alter AP shape at the 1° BP (axon) but slows and attenuates APs (grey dashes) in the terminal. All traces in this figure were obtained from the same terminal with K' based pipette solution and with Axopatch 200B.
Figure 2

**A1**

- Voltage steps of 40 mV, 20 mV, and 0 mV are applied to the membrane.
- The traces show the current response at 50 pA, 100 pA, and 2 ms.

**A2**

- Voltage steps of 40 mV, 20 mV, and 0 mV are applied to the membrane.
- The traces show the current response at 50 pA, 100 pA, and 2 ms.
- Inset: A single step from 0 mV to -20 mV.

**A3**

- Plot of membrane conductance (g) and capacitance (gmax) vs. membrane potential (Vm).
- Graph shows the relationship between iNa/Na(max) and Vm.

**B1**

- Voltage steps of 40 mV and -60 mV are applied to the membrane.
- The traces show the current response at 50 pA.

**B2**

- Plot of membrane current (iNa/Na(max)) vs. membrane potential (Vm).
- Graph shows the relationship between iNa/Na(max) and Vm.
- Arrow indicates Vm = -58 mV.

**C**

- Plot of membrane conductance (g) vs. membrane potential (Vm).
- Graph shows the relationship between g/gmax and Vm.