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tipE Regulates Na\(^+\)-dependent Repetitive Firing in Drosophila Neurons

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The tipE gene, originally identified by a temperature-sensitive paralytic mutation in Drosophila, encodes a transmembrane protein that dramatically influences sodium channel expression in Xenopus oocytes. There is evidence that tipE also modulates sodium channel expression in the fly; however, its role in regulating neuronal excitability remains unclear. Here we report that the majority of neurons in both wild-type and tipE mutant (tipE\(^{-}\)) embryo cultures fire sodium-dependent action potentials in response to depolarizing current injection. However, the percentage of tipE\(^{-}\) neurons capable of firing repetitively during a sustained depolarization is significantly reduced. Expression of a wild-type (tipE\(^{+}\)) transgene, in tipE\(^{-}\) neurons, restores repetitive firing to wild-type levels. Analysis of underlying currents reveals a slower rate of repolarization-dependent recovery of voltage-gated sodium currents during repeated activation in tipE\(^{-}\) neurons. This phenotype is also rescued by expression of the tipE\(^{+}\) transgene. These data demonstrate that tipE regulates sodium-dependent repetitive firing and recovery of sodium currents during repeated activation. Furthermore, the duration of the interstimulus interval necessary to fire a second full-sized action potential is significantly longer in single- versus multiple-spiking transgenic neurons, suggesting that a slow rate of recovery of sodium currents contributes to the decrease in repetitive firing in tipE\(^{-}\) neurons.

INTRODUCTION

Cell-specific changes in electrical excitability during early development are critical in formation of mature neural circuits (Spitzer et al., 1994). Modulation of neuronal excitability has also been implicated in mediating plasticity in the nervous system (Desai et al., 1999; Aizenman and Linden, 2000; Armano et al., 2000). Electrophysiological studies demonstrate that alterations in the number, type, localization, and/or posttranslational modifications of voltage-gated ion channels can influence neuronal excitability (Barish, 1986; Huguenard et al., 1988; O'Dowd et al., 1988; Spitzer, 1991; Turrigiano et al., 1995; Massengill et al., 1997; Catterall, 2000). However, the molecular mechanisms underlying regulation of excitability are less clear.

Using a genetic approach in Drosophila, progress has been made in identifying genes involved in mediating neuronal excitability. Shaker and ether-a-go-go (egg), mutants with hyperexcitable phenotypes, exhibit anomalous repetitive firing in larval motor axons and identify potassium channel genes important in determining the excitability properties of these neurons (Wu and Ganetzky, 1992; Littleton and Ganetzky, 2000). Temperature-sensitive paralytic mutants identify additional genes, such as paralytic (para) and no action potential (nap), that were recognized as playing a role in mediating neuronal excitability based on temperature-dependent blockade of action potential conduction in larval nerve fibers (Wu and Ganetzky, 1992). The para gene encodes a voltage-gated sodium channel \(\alpha\) subunit (Loughney et al., 1989), whereas nap encodes an RNA helicase involved in editing of para transcripts (Reenan et al., 2000).
tipE mutant flies, similar to nap and para mutants, exhibit a rapid and reversible temperature-sensitive paralysis (Kulkarni and Padhye, 1982). A reduction in the number of sodium channel binding sites in head membranes from tipE flies (Jackson et al., 1986) and a decrease in sodium current density in cultured tipE neurons (O’Dowd and Aldrich, 1988) suggest that tipE may regulate sodium channel expression. Enhanced temperature sensitivity for nerve conduction failure in para; tipE double mutants, compared with para alone, supports the suggestion that tipE can modulate axonal conduction properties (Ganetzky, 1986). The cloning of tipE revealed that the gene product is a novel integral membrane protein with two membrane spanning regions (Feng et al., 1995a). The tipE transgenic flies do not form a functional channel by itself when expressed in Xenopus oocytes but coexpression of tipE with para cRNA alters both the expression levels and the fast kinetic properties of the para-encoded voltage-gated sodium channels (Feng et al., 1995a; Warmke et al., 1997). Taken together these data suggest that tipE may define a new class of proteins that regulates electrical excitability through an interaction with the para sodium channel.

Analysis of excitability in tipE mutants has been limited to extracellular recordings in larval motor neurons that, interestingly, demonstrated apparently normal action potential propagation even at behaviorally nonpermissive temperatures (Ganetzky, 1986). Using cell cultures that contain subpopulations of primary Drosophila neurons exhibiting distinct firing phenotypes, we explored the role of tipE in regulation of neuronal excitability. A line of transgenic flies carrying the wild-type tipE gene under the control of a heat-shock promoter, in a tipE background (Feng et al., 1995b), was crucial in determining the electrical phenotypes associated with tipE. Our results demonstrate that tipE plays a role in regulating sodium-dependent repetitive firing properties in cultured Drosophila neurons.

RESULTS

tipE− Neurons Exhibit a Decrease in Repetitive Firing, Spontaneous Firing, and Action Potential Amplitude

To determine if the tipE gene plays a role in regulating neuronal excitability we compared the firing properties of embryonic tipE− and wild-type neurons grown in dissociated cell culture. Cultures from both genotypes contained heterogeneous populations of neurons: some with simple neurites and others with elaborately branched processes. Neurons could be found in clusters, where there was contact between neighboring cells (Figs. 1A and 1C), and in isolation (Figs. 1B and 1D). The whole-cell recording technique was used to examine branched neuritic processes in a wild-type (A) and a tipE− (C) culture. Isolated neurons in a wild-type (B) and a tipE− (D) culture. Cultures were grown for 2 days in vitro in DDM1, fixed in 4% paraformaldehyde, and stained with fluorescein-conjugated anti-HRP antibodies. Scale bars, 20 μm.

FIG. 1. Wild-type and tipE− Drosophila neurons grown in primary dissociated cell culture. Neuronal clusters interconnected by overlapping branched neuritic processes in a wild-type (A) and a tipE− (C) culture. Isolated neurons in a wild-type (B) and a tipE− (D) culture. Cultures were grown for 2 days in vitro in DDM1, fixed in 4% paraformaldehyde, and stained with fluorescein-conjugated anti-HRP antibodies. Scale bars, 20 μm.

The majority of neurons in both the tipE− (76 ± 5%, n = 10 platings) and the wild-type (82 ± 5%, n = 10 platings) cultures were electrically excitable based on the ability to elicit one or more action potentials in response to a 600-ms, suprathreshold depolarizing current step. The electrically excitable neurons were grouped into three broad classes: single spiking, graded multiple spiking, or multiple spiking (Figs. 2A and 2B). The single spiking neurons were characterized by a single action potential elicited at the beginning of the step polarizations in the
amplitude of current injected were capable of inducing additional action potentials during the 600-ms step. The graded multiple-spiking neurons fired two to six action potentials at the beginning of a 600-ms depolarizing current step with a steady decrement in amplitude of each successive action potential following the first one or two. In these neurons no spikes were elicited after the first 300 ms (Fig. 2A).

The third class, multiple-spiking neurons, fired trains of action potentials throughout the 600-ms depolarizing current step and each action potential was of approximately equal amplitude (Fig. 2B). The multiple-spiking category could be further subdivided into three subclasses (tonic, adaptive, and delayed) as previously described in “giant” Drosophila neurons grown in cell culture (Zhao and Wu, 1997). The tonic firing pattern was characterized by a relatively constant interspike interval during a train of action potentials (Fig. 2B). The adaptive subclass was characterized by a decrease in frequency during the spike train. Neurons were included in this class if the first interspike interval was less than 70% of the last interspike interval in the train. Some, but not all, adaptive cells contain a doublet at the beginning of the spike train as seen in the adaptive cell shown in Fig. 2B. In the delayed subclass, action potentials were usually observed after a latency of >100 ms from the onset of the stimulus (Fig. 2B). However, in a small number of cells in this group the delay was <100 ms but the interspike interval became progressively shorter throughout the action potential train. Repeated depolarizing stimuli, separated by 2–5 s at rest, resulted in reproducible firing patterns in neurons within each of the three classes.

Spontaneous action potentials were also observed in some of the cultured cells. Neurons were classified as spontaneously firing if: (1) action potentials were observed in extracellular recordings obtained in the cell-attached configuration and/or (2) action potentials were observed in intracellular current clamp recordings at the cell’s resting potential in the absence of step depolarizations (Fig. 2C).

Neurons in all three firing classes were observed in both wild-type and tipE- cultures. However, there was a significant difference in the distribution of neurons within these three classes (Fig. 3). In wild-type cultures the most prevalent class of neurons was multiple spiking, comprising approximately 70% of all electrically excitable cells, with the single-spiking class representing about 25% of the total. In contrast, single-spiking neurons were the most abundant class (50% of total) in the tipE- cultures, with only 25% of the neurons exhibiting multiple-spiking properties (Figs. 3A and 3B). Among tipE- neurons that were multiple spiking, the distribution within the three subclasses was similar to that of wild type (Table 1). Spontaneously firing neurons were observed in both tipE- and wild-type cultures. However, there was a twofold decrease in the incidence of spontaneously firing neurons in the tipE- cultures compared with wild type (Fig. 3C). Because the vast majority of spontaneously firing neurons detected were in the multiple-spiking class, this decrease is likely to reflect the reduction in the percentage of multiple-spiking neurons in the tipE- cultures.

Analysis of the first spike induced by suprathreshold depolarization in each neuron revealed that the action

**FIG. 2.** Embryonic Drosophila neurons grown in DDM1 exhibit heterogeneous firing properties. (A) Representative whole-cell current clamp recordings obtained from two neurons illustrating the single-spiking and the graded multiple-spiking firing classes. (B) The multiple-spiking class is composed of three subclasses; examples of each type are illustrated. Voltage traces in both A and B were recorded in response to a 600 suprathreshold depolarizing current step from a negative holding potential. (C) Spontaneous action potentials recorded from a single neuron, first in a cell-attached recording configuration (extracellular), followed by recordings made in the whole-cell recording configuration (intracellular), in the absence of depolarizing current injection. All electrophysiological recordings in these and the subsequent figures were obtained at room temperature from neurons at 2–3 days in vitro.
The reduced repetitive firing, spontaneous firing, and action potential amplitude in tipE mutant neurons are consistent with the hypothesis that tipE regulates these properties in Drosophila neurons. However, one caveat to this interpretation is that the comparison was made between populations of neurons harvested from two fly strains in which the contribution of differences in genetic backgrounds is unknown. To determine if the altered electrical properties in tipE neurons are the consequence of a mutation in the tipE gene we studied a transgenic line (tipE:tipE⁺) containing the wild-type tipE transgene (tipE⁺), under the control of a heat shock promoter, in the tipE⁻ background.

To monitor expression of the transgene and determine if it could be regulated in the neurons by heat shock, cultures were prepared from wild-type, tipE⁻, and tipE⁺:tipE⁻ embryos. Half of the cultures in each genotype were subjected to three 1-h heat shocks (see Experimental Methods for details). Non-heat-shocked cultures were maintained continuously at room temperature. RNA was prepared from all the cultures at 42 h after plating. Primers (M1 and M2) flanking a single point mutation in the tipE cDNA (removing an RsaI restriction enzyme site) generated PCR products of distinct sizes in wild-type and tipE⁻ neurons (Fig. 4A). In this analysis we found that wild-type and tipE⁻ neurons express both mutant and wild-type mRNA, even in the absence of heat shock (Fig. 4B). The relative abundance of the wild-type tipE product in the tipE⁺:tipE⁻ cultures was dramatically increased (>10-fold) following heat shock. However, since the absolute levels of tipE mRNA produced are not known it was possible that, even in the absence of heat shock, wild-type tipE levels might be sufficient to rescue the mutant firing phenotype. Therefore, in all the electrophysiology

### TABLE 1

<table>
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<th>Subclass Distribution of Multiple Spiking (MS) Neurons</th>
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<tr>
<td>Tonic (%MS)</td>
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<tr>
<td>Wild type</td>
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<tr>
<td>tipE⁻</td>
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Note. 9 platings; mean ± SEM.
studies comparisons included analysis of neurons in tipE⁻:tipE⁺ cultures examined after heat shock and in sibling cultures that were maintained continuously at ambient temperature.

To address the role of tipE in neuronal excitability, independent of development, tipE⁻:tipE⁺ cultures were grown in the absence of heat shock for the first 2 days in vitro, by which time differences in the three major firing classes were readily apparent between tipE⁻ and wild-type neurons. In four independent experiments, half of the tipE⁻:tipE⁺ cultures were exposed to two 1-h heat shocks at 42 and 49 h after plating while the remaining cultures were not heat shocked (Fig. 5, top). To control for the affects of heat shock alone, wild-type cultures prepared in parallel were exposed to the same heat-shock regime. Cultures were coded and examined blind with respect to genotype and heat-shock conditions. Examples of firing properties in three different neurons recorded from a wild-type (+HS), a tipE⁻:tipE⁺ (−HS), and a tipE⁻:tipE⁺ (+HS) culture are illustrated in Fig. 5A. In the absence of heat shock, there were very few multiple-spiking neurons in the tipE⁻:tipE⁺ cultures, with the majority of excitable cells split between the graded multiple-spiking and single-spiking firing classes (Fig. 5B), similar to the distribution seen previously in the tipE⁻ cultures. These data demonstrate that the level of wild-type tipE product in the absence of heat shock does not rescue the mutant firing phenotype in transgenic cultures. However, the altered firing type distribution was fully rescued within 24 h after heat shock (+HS), with over 90% of the neurons in the multiple-spiking firing class and the remainder classified as single spiking, similar to the distribution seen in wild-type cultures (+HS) examined in parallel (Fig. 5B). The percentage of spontaneously firing neurons, low in the tipE⁻:tipE⁺ cultures in the absence of heat shock (−HS), was also rescued following heat shock (+HS) (Fig. 5C). Unexpectedly, the reduced action potential amplitude in the transgenic neurons was not rescued following heat shock (Fig. 5D).

### TABLE 2
Comparison of Electrical Properties in Wild-Type and tipE⁻ Neurons at 2–3 DIV

<table>
<thead>
<tr>
<th></th>
<th>Action potential duration (ms)</th>
<th>Input resistance (GOhm)</th>
<th>Resting potential (mV)</th>
<th>Capacitance (pA)</th>
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<tr>
<td>Wild type</td>
<td>6.5 ± 1.2 (35)</td>
<td>1.3 ± 0.1 (43)</td>
<td>−38.0 ± 2.0 (53)</td>
<td>22.3 ± 1.3 (45)</td>
</tr>
<tr>
<td>tipE⁻</td>
<td>6.7 ± 1.0 (33)</td>
<td>1.4 ± 0.2 (46)</td>
<td>−37.0 ± 2.1 (43)</td>
<td>19.0 ± 1.2 (54)</td>
</tr>
</tbody>
</table>

*Note. Mean ± SEM (No. of neurons).*

**FIG. 4.** Heat shock drives expression of wild-type tipE mRNA in a tipE⁻ background. (A) Schematic representation of the tipE cDNA with the orientation of primer pair M1 (radioactively labeled) and M2. Positions of two RsaI restriction enzyme sites, R1* (eliminated in the tipE mutant) and R2 are shown. RsaI digestion of the PCR product generated by amplification using M1/M2 yields a labeled fragment of 85 bp in tipE⁻ and 66 bp in wild type. (B) Autoradiogram of RsaI digests of PCR products from wild-type, tipE⁻, and tipE⁻:tipE⁺ (±HS) cultures. Only the 66-bp product is amplified in RNA prepared from wild-type neurons. Amplification of only the 85-bp product is observed in RNA prepared from tipE⁻ neurons. Heat shock does not alter expression of these products in either the wild-type or the tipE⁻ neurons. RNA prepared from tipE⁻:tipE⁺ transgenic embryo cultures expresses both the wild-type and the mutant product even in the absence of heat shock. However, following heat shock there is a dramatic increase in the relative abundance of the wild-type (66 bp) versus the mutant (85 bp) message. Cultures were heat shocked by incubation in a 37°C, 5% CO₂ incubator for 1 h at 16, 33, and 40 h after plating (see Experimental Methods for details). Total RNA was extracted 2 h after the last heat shock.

**Repolarization-Dependent Recovery of Sodium Currents and Excitability in Wild-Type, Mutant, and Transgenic Neurons**

Previous studies had demonstrated that the wild-type tipE gene product upregulates the amplitude and alters the kinetic properties of para sodium currents in a
heterologous expression system (Feng et al., 1995a; Warmke et al., 1997). In addition, a decrease in sodium current density in \textit{tipE}^{-} versus wild-type neurons was observed at 1 day in culture (O’Dowd and Aldrich, 1988). Therefore, we asked if there were changes in the sodium current properties that might contribute to the mutant firing phenotypes seen in the present culture condition. These studies were complicated by the fact that the sodium currents in the majority of the electrically excitable neurons could not be well voltage-clamped, precluding a classical quantitative biophysical analysis of the underlying sodium channel properties. However, comparison of features of the whole-cell currents elicited by step depolarizations, using identical recording conditions for wild-type, mutant, and transgenic neurons, allowed us to identify sodium current properties linked to \textit{tipE} expression.

The first comparison focused on the maximal sodium current activated in mutant and wild-type neurons. Individual neurons were stimulated with a series of increasing depolarizing voltage steps in the presence of cesium in the internal solution to block outward potassium currents. The sodium current density in each neuron was determined by normalizing the peak inward sodium current to the whole-cell capacitance. There was a 25\% reduction in the peak sodium current density in the \textit{tipE}^{-} versus wild-type neurons (Fig. 6A). The sodium current density in the transgenic neurons in the absence of heat shock was similarly low. However, this reduced sodium current density was not rescued following heat shock (Fig. 6A). The inability to rescue sodium current density is consistent with the inability to rescue the action potential amplitude, suggesting that these two properties are linked. These data further demonstrate that recovery of robust repetitive firing does not require rescue of the sodium current density.

In light of our finding that most \textit{tipE} mutant neurons are capable of firing a single action potential but are compromised in their ability to fire repetitively, we investigated the recovery of sodium currents during repetitive activation. Neurons were subject to two identical voltage steps from $-75$ to $-5$ mV, separated by a 10-ms interstimulus interval at $-75$ mV (Fig. 6B). The amplitude of the current elicited by the second step was not rescued by induction of the \textit{tipE}^{+} transgene: there is no significant difference between the mean amplitude in neurons in $+\text{HS}$ ($n = 13$) and $-\text{HS}$ ($n = 12$) \textit{tipE}^{+}/\text{HS}$ cultures ($P > 0.05$, Student’s t test). Bars on all graphs indicate SEM.
in the mutant was also seen in the transgenic neurons in the absence of heat shock. Following heat shock, sodium current recovery was restored to wild-type levels (Fig. 6B). There was no decrement in sodium current amplitude following a 2-s interstimulus interval at −75 mV, the standard time between repeated trials, in either mutant or rescued transgenic neurons. These findings demonstrate that the tipE mutation results in a slower rate of repolarization-dependent recovery of sodium currents during repetitive activation.

A straightforward interpretation of these data would be that the tipE mutation slows but does not block recovery of the underlying sodium channels from inactivation. In the absence of excess sodium channels, this would decrease the probability of repetitive spiking during a sustained depolarization. The poor space clamp in the cultured neurons makes a more detailed investigation of the kinetics of recovery of sodium currents from inactivation problematic. However, if slowed recovery from inactivation contributes to the tipE firing phenotype, then one would predict that repolarization should be necessary, and 2 s interstimulus interval sufficient, for firing a second spike in tipE (single-spiking) neurons. Therefore, five single-spiking neurons, in transgenic cultures in the absence of heat shock, were held between −50 and −60 mV and subjected to prolonged depolarizing steps (5 s) separated by 2 s intervals. All neurons fired one action potential at the onset of each depolarizing step. These data demonstrate that an interstimulus interval is necessary and 2 s is sufficient for firing a second full-sized action potential in tipE neurons that are classified as single spiking.

The relationship between interstimulus duration and recovery of excitability was examined in cultures of transgenic neurons, half that were heat shocked and half that served as controls. Neurons were held at voltages between −50 and −60 mV and given two identical, suprathreshold, depolarizing, current steps. The step depolarizations were separated by intervals of varying duration (Fig. 7A). An example of the typical behavior of a single-spiking transgenic neuron, in the absence of heat shock, is shown in the top traces in Fig. 7B. The second action potential was reduced in amplitude when the two depolarizing pulses were separated by short interstimulus intervals of 2 and 10 ms (first and second pair). The action potential amplitude was similar in the two steps when the interstimulus interval was increased to 100 ms (third pair, Fig. 7B). In contrast, in a multiple-spiking transgenic neuron, following heat shock rescue, there was no significant decrement in the amplitude of the action potential elicited by a second pulse.
step depolarization, even when the interstimulus interval was as short as 2 ms (Fig. 7B, bottom). To quantify these data, the action potential amplitude elicited by the second step was normalized to that elicited by the first step and plotted as a function of interstimulus interval, for a number of neurons in each firing class (Fig. 7C). As illustrated in the graph, the interstimulus duration required for recovery of the ability to fire a full-sized action potential was longer in the single-spiking transgenic neurons. In graded multiple-spiking neurons the required interstimulus duration was intermediate to the other two classes. The longer interstimulus duration necessary to fire a second full-sized action potential in single- versus graded versus multiple-spiking classes is consistent with the suggestion that a slowed rate of repolarization-dependent recovery of sodium currents contributes to the decrease in repetitive firing in tipE− neurons.

In addition to sodium currents, voltage-gated potassium currents are critical in determining many aspects of neuronal excitability, including repetitive firing properties (Wu and Ganetzky, 1992). Potassium currents were induced by a series of 300-ms depolarizing voltage steps between −55 and +55 mV. Individual neurons in both genotypes had varying levels of transient and sustained currents. The peak and plateau current amplitudes in wild-type and tipE− neurons were determined at +55 and normalized to the whole-cell capacitance. There was no significant difference in the peak (wt 63.9 ± 8 pA/pF, n = 25; tipE− 62.6 ± 4 pA/pF, n = 48) nor in the plateau current density (wt 33.5 ± 3 pA/pF, n = 22; tipE− 35.1 ± 2.5 pA/pF, n = 47) between the two genotypes. Since there were no prior studies indicating that tipE regulates potassium channels, we did not compare the properties of voltage-gated potassium currents in tipE− and wild-type neurons further.

Coexpression of tipE and para mRNA in Wild-Type Neurons

Previous studies from our laboratory have demonstrated that the para gene encodes functional sodium channels in neurons cultured from wild-type Drosophila embryos (O’Dowd et al., 1989). If tipE is regulating para expression, thereby influencing sodium currents and firing properties in Drosophila neurons as suggested from the oocyte studies, then tipE and para must be expressed at the same time and in the same cells. RT-PCR with two distinct primer sets (see Experimental Methods) was used to examine expression of para and

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**FIG. 7.** Recovery of excitability in transgenic neurons. (A) Neurons in transgenic cultures were held at −55 mV and stimulated with the two-step protocols illustrated using three interstimulus intervals (2, 10, 100 ms). (B) Whole-cell current clamp recordings from a single-spiking neuron in the absence of heat shock (tipE−:tipE− HS) (top trace) and a multiple-spiking neuron in the presence of heat shock (tipE−:tipE− +HS) (bottom trace). In the single-spiking neuron the action potential elicited by the second step in each pair was reduced in amplitude for the interstimulus intervals of 2 and 10 ms. When the interstimulus interval was extended to 100 ms the second action potential was similar in amplitude to the first. In a multiple-spiking neuron the action potential had been heat shocked, there was no difference in action potential amplitude in the first and second steps even when the interstimulus interval was 2 ms. (C) The amplitude of the first action potential (measured from peak to trough) generated in the second step in each pair was normalized to the amplitude of the first action potential in the first step and plotted as a function of interstimulus interval. Multiple-spiking neurons showed no decrease in the normalized action potential amplitude at any of the interstimulus intervals examined. In contrast, the normalized action potential amplitude was significantly reduced in graded multiple spiking and even further reduced in single-spiking neurons at intervals shorter than 20 ms. The values for single- and multiple-spiking classes, between 2 and 12 ms, when evaluated in a point-by-point comparison are significantly different (ANOVA, P < 0.001 (2–8 ms), P < 0.01 (10–12 ms), Fisher’s protected least significant difference). Each data point represents the mean value for the indicated number of neurons in each firing class. Bars indicate SEM.
**DISCUSSION**

Although little is known about the firing properties of *Drosophila* neurons in vivo (Ikeda and Kaplan, 1970; Tanouye *et al.*, 1981) studies in cell culture reveal subpopulations of neurons with distinct firing patterns elicited by depolarizing current pulses (O’Dowd, 1995; Zhao and Wu, 1997). Many of the multiple-spiking neurons in this study have spike discharge patterns that closely resemble those of the well-characterized regular-spiking or fast-spiking firing classes of mammalian
cortical neurons described in the animal and in dissociated cell culture (Connors and Gutnick, 1990; Massengill et al., 1997). The single-spiking neurons in the Drosophila cultures are similar to the recently described on-spiking neurons found in the rodent auditory cortex, which fire only one or two spikes that occur within 10 ms of the onset of a maintained intracellular depolarization (Metherate and Aramakis, 1999). The presence of similar firing classes in the cultured Drosophila neurons and rodent cortical neurons suggests strong conservation of the functional elements contributing to CNS circuitry between these distantly related species.

It should also be noted that the resting potentials of the Drosophila neurons reported in this study are more depolarized than is standard for many mature mammalian neurons. However, hyperpolarizing shifts in membrane potential, from −40 to −65 mV, have been reported during early development in some populations of mammalian cortical neurons (Agmon et al., 1996; Zhou and Hablitz, 1996). This suggests that the depolarized resting potentials could be related to the relatively young age at which most of the recordings were obtained, 2–3 days of the neuronal birth date. More negative resting potentials of −55 mV have been reported for Drosophila “giant neurons” examined at slightly later stages, between 2 and 5 days in culture (Yao and Wu, 1999). In addition, we have observed more hyperpolarized resting potentials (−55 mV) when recordings are done at 3–4 days (unpublished data).

Despite the depolarized resting potentials, intracellular (whole cell) recordings revealed spontaneous action potentials in the absence of current injection in some neurons. This does not seem likely to be injury-induced spiking as spontaneously active neurons were observed at a similar frequency in extracellular (cell attached) recordings. Previous studies from our lab have also demonstrated the presence of action potential mediated spontaneous excitatory postsynaptic currents in many of these cultured neurons, in which activity in the presynaptic neuron is clearly independent of technical artifacts that could be potentially associated with whole-cell recording electrodes (Lee and O’Dowd, 1999). Finally, recordings from neurons in the Drosophila embryonic nerve cord have revealed large spontaneously active currents, thought to underlie action potentials, in neurons held at −40 mV, that were rarely seen in those held at more hyperpolarized potentials (Baines and Bate, 1998). Together these findings suggest that young embryonic Drosophila neurons, both in vivo and in vitro, are excitable at relatively depolarized voltages.

**tipE Regulates Sodium-Dependent Repetitive Firing**

Assessment of the firing properties in primary neurons from genetic mutants is a useful strategy for examining the role of specific genes in regulating neuronal excitability. Alterations in spontaneous activity of neurons cultured from Hyperkinetic mutant embryos (Yao and Wu, 1999) supported an early study indicating that this gene, encoding a K channel β subunit, is involved in regulation of neuronal firing properties (Ikeda and Kaplan, 1970). Our analysis of tipE− neurons revealed reductions in repetitive firing, spontaneous firing, action potential amplitude, peak sodium current density, and sodium current recovery during repeated activation, suggesting that these are linked to each other and to tipE. Rescue experiments, involving expression of the wild-type tipE transgene in tipE− neurons, confirmed that tipE is important in regulation of repetitive firing, spontaneous firing, and the rate of recovery of sodium currents during repeated activation. Our data also demonstrate that induction of tipE+ expression in transgenic neurons beginning at 2 days, after neurons have already established their firing properties, is sufficient to rescue the mutant firing phenotypes. This suggests that regulation of tipE may play a role, not only in establishment of neuronal firing phenotype, but also in modulation of firing properties in differentiated neurons.

The slower rate of recovery of sodium currents during repetitive activation in tipE− neurons predicts that a diminished sodium current will be available for generation of the second spike in an action potential train in the mutant neurons. This could thus contribute to the decrease in probability of mutant neurons firing repetitively during sustained depolarization. Concomitant rescue of sodium current recovery and repetitive firing, following induction of the tipE+ transgene in tipE− neurons, suggests linkage between these two phenotypes. The difference in the level of recovery of sodium currents during repolarization seen between wild-type and mutant neurons, though significant, was not large (approximately 10%), and therefore it was not clear how this property might influence repetitive firing rates. However, analysis of the recovery of excitability as a function of interstimulus interval in the different firing classes is consistent with the suggestion that reduced rate of recovery of sodium currents contributes to the decrease in repetitive firing in mutant neurons. In single-spiking neurons, an interstimulus interval was required for recovery of the ability to fire a second action.
potential. In addition, the duration of the interstimulus interval necessary to fire a second full-sized action potential was significantly longer in single- versus multiple-spiking transgenic neurons.

In Drosophila, as in mammals, the sodium channels that underlie the whole-cell sodium currents are transiently activated by a sustained depolarizing voltage step and recovery from inactivation requires return to hyperpolarized potentials (O’Dowd and Aldrich, 1988). A decrease in the rate of recovery from inactivation of the underlying sodium channels is one mechanism that could contribute to the reduced recovery of sodium currents seen in the tipE neurons. Studies in other systems have clearly demonstrated a relationship between rate of recovery of sodium channels from inactivation and repetitive firing. In hippocampal pyramidal neurons spikes in the dendrites are attenuated by high-frequency stimulation and this is correlated with a relatively slow rate of recovery of sodium channels from inactivation (Colbert et al., 1997; Jung et al., 1997). A computational model supports the hypothesis that delayed recovery of sodium channels from inactivation can result in attenuation of action potentials (Migliore, 1996). Additionally, hyperexcitability characterized by elevated firing frequencies in spinal sensory neurons following injury has been associated with the emergence of sodium currents that recover rapidly from inactivation (Cummins and Waxman, 1997; Cummins et al., 2000). However, in the present study the majority of the data on sodium currents were obtained from neurons that could not be well voltage-clamped. Therefore, we cannot rule out the possibility that a use-dependent change in space constant, rather than a change in the sodium channel inactivation properties, could contribute to the observed decrease in recovery of the currents. For example, a failure to reach the same membrane potential during the two sequential depolarizing steps could cause a reduction in amplitude of the sodium current evoked by the second pulse. We do not believe this was a factor since the latency and waveform of the currents, also influenced by space constant, did not vary significantly between the two steps (Fig. 6B). In addition, for this mechanism to account for the differences seen between tipE- and wild-type neurons and the rescue by tipE+, it would necessitate invoking genotype-specific differences in the properties of use-dependent alterations in space clamp. In either case, our rescue studies clearly demonstrate that tipE is important for regulating recovery of sodium currents from repeated activation and sodium-dependent repetitive firing. Therefore, isolation of vertebrate tipE orthologues may identify novel pathways involved in regulation of sodium currents that can influence action potential propagation in mammalian neurons.

Most of the spontaneously firing neurons in wild-type cultures were in the multiple-spiking class. Alterations that decrease the probability of firing a second spike in the mutant neurons in response to depolarization could also decrease the probability of firing spontaneously. However, additional changes in the underlying currents may contribute to the reduced spontaneous activity in the mutant neurons. For example, in oocytes, coexpression of the wild-type tipE product influenced both the density and the fast decay kinetics of the para sodium currents (Warmke et al., 1997). The fast kinetic properties of sodium currents were not assessed in the present study due to inadequate voltage-clamp in excitable cells. Therefore, tipE might also affect fast gating properties of sodium channels that could contribute to the altered firing phenotypes observed.

The oocyte studies further suggested that tipE might be functioning like sodium channel β subunits (β1 and β2) as these are known to influence both expression levels and fast kinetic properties of mammalian sodium channels (Isom et al., 1994). A newly identified β subunit (β3), cloned from human and rat, has been shown to influence the rate of sodium current recovery from inactivation (Morgan et al., 2000), similar to the role suggested for tipE by the present study. Our single-cell RT-PCR analyses demonstrate that tipE is coexpressed with para in most cells, and coimmunoprecipitation in Xenopus oocytes suggests that the two proteins can physically associate (L. M. Hall and C. Ericsson, unpublished results). Taken together these data suggest that, although tipE has little amino acid sequence identity with sodium channel β subunits, it could be functioning as an auxiliary subunit important in regulating sodium channel function in wild-type Drosophila neurons. A prediction of this hypothesis is that wild-type neurons that fire multiple spikes express more tipE than those that fire only single action potentials. A quantitative analysis of gene and/or protein levels, not undertaken in the present studies, would be necessary to address this question.

The inability of wild-type tipE transgene expression to rescue the reduced sodium current density and action potential amplitude in transgenic neurons was surprising. It is possible that these features, while related to each other, are not necessarily linked to tipE. However, we cannot rule out the possibility that tipE plays a role in regulation of sodium current density and action potential amplitude in primary neurons. For example,
induction conditions or the timing of the assay could be suboptimal for detecting regulation mechanisms involving coassembly of tipE products with para sodium channels prior to membrane insertion. In either case, rescue of the repetitive firing phenotype in the absence of restoration of sodium current density and action potential amplitude demonstrates that these can be functionally separated.

**tipE** \(^+\) Is Not Necessary for Repetitive Spiking in All Drosophila Neurons

Repetitive firing, spontaneous activity, and fast recovery of sodium currents from repeated activation in some of the tipE \(^-\) neurons demonstrate that tipE \(^+\) expression is not necessary for manifestation of these electrophysiological phenotypes in all cultured neurons. It is possible that a tipE homologue, identified in a recent analysis of the Drosophila genome (Littleton and Ganetzky, 2000), encodes a protein that substitutes for Drosophila Hypomorph rather than a true null (Feng et al., 1992) resulting in a premature stop codon, may act as a hypomorphic rather than a true null (Feng et al., 1995b). The tipE \(^-\) neurons with apparently wild-type properties could be due to residual function of the mutant protein. This second possibility seems less likely because previous studies have shown that mutant tipE \(^-\) was not able to rescue adult paralysis (Feng et al., 1995b). Additionally, mutant tipE cRNA expressed in Xenopus oocytes does not enhance para sodium current expression (M. Chopra and L. M. Hall, unpublished observations).

**Functional Significance**

In nap and para mutants, a temperature-dependent blockade of action potential propagation in larval motor nerves has been associated with the temperature-sensitive paralysis (Wu and Ganetzky, 1992). In contrast, it was unclear why tipE \(^-\) larvae exhibit normal extracellularly recorded action potential propagation in motor nerves both at the behaviorally permissive and at the nonpermissive temperature (Ganetzky, 1986). Our data demonstrate that tipE \(^-\) neurons are capable of generating a single action potential in response to a discrete stimulus, consistent with the apparently normal compound action potential recorded in larval motor neurons. However, our findings suggest that action potential propagation in tipE \(^-\) mutant nerves could be compromised during high-frequency, repetitive nerve stimulation due to the reduced sodium current density and depressed recovery of sodium currents during repeated stimulation. Furthermore, a rise in temperature speeds up the gating kinetics of all channels and may result in potassium currents overwhelming the altered sodium currents leading to an even more pronounced alteration at elevated temperatures. This could contribute to the temperature-induced paralysis. The reduced sodium current density and altered repolarization-dependent sodium current recovery in tipE \(^-\) mutant neurons could also contribute to the enhanced sensitivity to temperature-induced action potential blockade previously reported in the para;tipE double mutants (Ganetzky, 1986).

**EXPERIMENTAL METHODS**

**Drosophila stocks and cell culture.** Embryos were collected from Canton-S homozygous wild-type, tipE sepia (tipE \(^-\) ), and w; tipE sepia flies transformed with a wild-type tipE cDNA under control of the heat-shock promoter (tipE :tipE \(^-\) ) (Feng et al., 1995b). Neurons were prepared from midgastrula-stage embryos and cultured in Drosophila defined medium 1 (DDM1) at 22–25°C and 4–5% CO\(_2\), as previously described (O’Dowd, 1995). Cultures stained with anti-horseradish peroxidase (HRP) antibodies were fixed in 4% paraformaldehyde for 30 min at room temperature followed by a 1-h incubation with fluorescein-conjugated anti-HRP antibodies (1:100; Organon Teknika). Coverslips were mounted on glass slides. Images were acquired with a Spot cooled CCD camera (Diagnostic Instruments) mounted on a Nikon Optiphot microscope and prepared for presentation in Adobe PhotoShop.

**Electrophysiological recordings.** To minimize potential bias in selection of cells for analysis, whole-cell recordings from wild-type, tipE \(^-\) , and tipE :tipE \(^-\) transgene neurons were performed blind with respect to genotype and heat-shock treatment. Unpolished recording pipettes had open pipette resistances of 2–5 MΩ. For assessment of firing properties the internal pipette solution contained (in mM): potassium gluconate (120), NaCl (20), EGTA (1.1), CaCl\(_2\) (0.1), MgCl\(_2\) (2), Hepes (10), pH 7.2. Sodium currents were examined using an internal solution containing (in mM): d-gluconic acid (120), cesium hydroxide (120), NaCl (20), CaCl\(_2\) (0.1), MgCl\(_2\) (2), EGTA (1.1), Hepes (10), pH 7.2. The external solution contained (in mM): NaCl (140), KCl (3), MgCl\(_2\) (4), CaCl\(_2\) (1), Hepes (5), pH 7.2. A 5-mV liquid junction potential has been subtracted from all
membrane potentials noted in this report. Whole-cell capacitance was determined by measuring the area under the capacitative transient current record obtained immediately after break into the cell. Data were collected and analyzed using a List EPC-7 patch-clamp amplifier, a Dell computer, and pCLAMP software (Axon Instruments). All recordings were performed at room temperature.

**Heat-shock induction of tipE expression in transgenic neurons.** Cultures were prepared from midgastrula-stage embryos obtained from wild-type, tipE+, and tipE− tipE flies. For PCR analysis of gene expression, half of the cultures from each genotype were heat shocked by transfer to a 37°C, 5% CO2 incubator for 1 h at 16, 33, and 40 h after plating. The remainder of the time they were maintained at ambient temperature (22–25°C). The sibling cultures were maintained continuously in a 5% CO2 incubator at ambient temperature for 42 h. Total RNA was extracted at 42 h (2 h after the last heat shock) from both control and heat-shocked cultures. For the electrophysiological studies, half of the cultures from each genotype were heat shocked in a 5% CO2 incubator for 1 h at 42 and 49 h after plating. The remainder of the time they were maintained in a 5% CO2 incubator at ambient temperature. The sibling cultures were maintained continuously at ambient temperature. All electrophysiological recordings were done at 66–74 h after plating.

**RT-PCR analysis of gene expression in cultured neurons.** Total RNA from cultured neurons was prepared using Tri-Reagent (Molecular Research Center, Inc., Cincinnati, OH) according to a single-step method (Chomczynski and Sacchi, 1987). First-strand cDNA was generated by random-primed reverse transcription of total RNA, and PCR amplification of the cDNA was performed as previously described (O’Dowd et al., 1995) using the primer pairs shown in Table 3. Amplified products, visualized by inclusion of 2–5 × 104 dpm of 32P-end-labeled forward primers in the PCR, were separated by electrophoresis on 8 or 10% nondenaturing polyacrylamide gels. The amount of product was quantified by phosphorimager analysis (Molecular Dynamics, Sunnyvale, CA).

Identification of wild-type and mutant tipE PCR products was performed by RsaI restriction enzyme analysis of an aliquot of the PCR products using standard procedures (Sambrook et al., 1989). In the developmental study a single reverse transcription reaction was performed on each RNA sample for each time point. This was divided into three equal aliquots in which PCR products were amplified using primers specific for ribosomal protein 49 (rp49, 21 cycles) or para or tipE (25 cycles). Cycle numbers were chosen to yield products within the linear range of amplification. To minimize differences in reaction conditions, primers of similar size and specific activities were used. Phosphorimager optical density measurements for developmentally regulated PCR products were normalized to optical density values obtained from PCR amplification of rp49, a mRNA that is not developmentally regulated (O’Connell and Rosbash, 1984). Single-cell amplification of total RNA aspirated from neurons after electrophysiological recordings was performed as previously described (O’Dowd et al., 1995).

**Primer pairs.** para: To amplify a single product common to all para transcripts the primer set paraComF/R was used. To examine the distribution of para transcripts containing alternatively spliced exons a and i, a primer pair (paraDP3/DP4) flanking these exons was used. tipE: For developmental profiles and single-cell experiments, PCR amplification of tipE mRNA was performed using the primer pair tipEComF/R. To dif-

### TABLE 3

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<th>Primer name</th>
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<th>Nucleotide positions</th>
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