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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 *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.

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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* (*eag*), 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 α subunit (Loughney *et al.,* 1989), whereas *nap* encodes an RNA helicase involved in editing of *para* transcripts (Reenan *et al.,* 2000).

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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* protein does 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 the firing properties of neurons at 2 and 3 days *in vitro.* All recordings were performed blind with respect to genotype. Initial studies were conducted to determine if a correlation between morphological features and neuronal excitability could be established. Because there did not appear to be systematic differences in the electrical properties of isolated versus clustered neurons all data were grouped for statistical analysis.

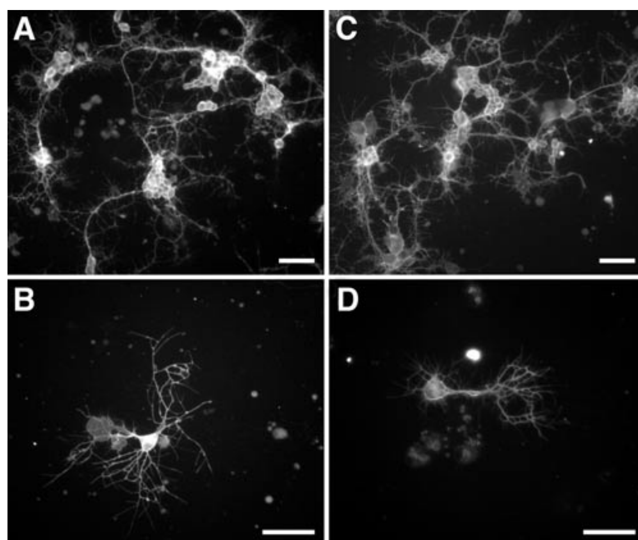

FIG. 1. Wild-type and *tipE*⁻ *Drosophila* neurons grown in primary dissociated cell culture. Neuronal clusters interconnected by overlapping branched neuritic processes in a wild-type (A) and a *tipE*⁻ (C) culture. Isolated neurons in a wild-type (B) and a *tipE*⁻ (D) culture. Cultures were grown for 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 depolarization (Fig. 2A). In this class of neurons, neither changes in holding potential nor increases in the

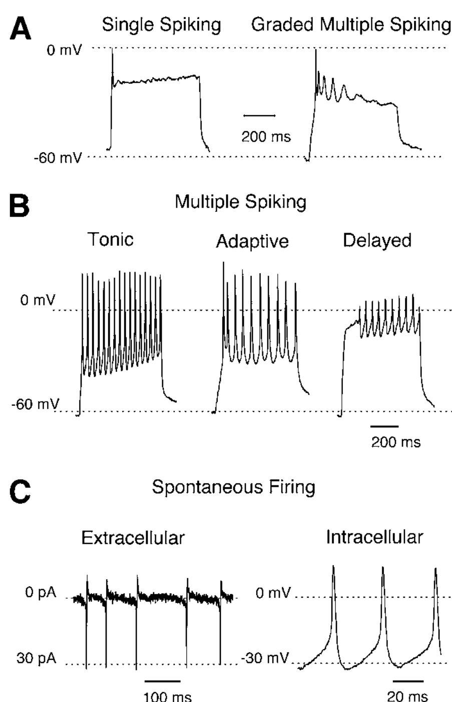

FIG. 2. Embryonic *Drosophila* neurons grown in DDM1 exhibit heterogeneous firing properties. (A) Representative whole-cell current clamp recordings obtained from two neurons illustrating the single-spiking and the graded multiple-spiking firing classes. (B) The multiple-spiking class is composed of 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.*

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

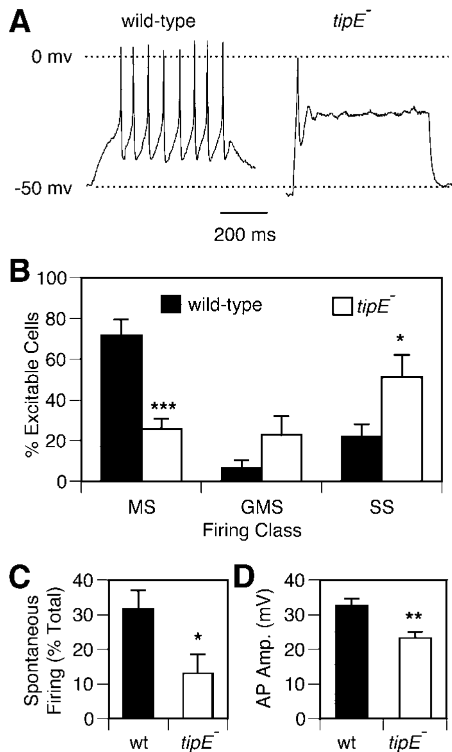

FIG. 3. The frequencies of repetitive firing, spontaneous firing, and action potential amplitude are decreased in *tipE*⁻ neurons. (A) Representative recordings illustrating the most abundant firing class in wild-type and *tipE*⁻ neurons. (B) There was a significant reduction in the percentage of neurons in the multiple-spiking (MS) class in *tipE*⁻ cultures compared with wild-type, with a corresponding increase in the % of neurons in the single-spiking (SS) class. The mean percentage of total excitable cells within each firing class was determined by calculating the percentage observed in nine separate experiments in which three or more neurons were examined. (C) The fraction of neurons firing spontaneous action potentials, as a function of the total number of neurons examined, was significantly lower in *tipE*⁻ compared with wild-type. The means represent data obtained from three or more neurons in four separate platings for both genotypes. (D) The amplitude of the first action potential (AP) in each train (peak to trough) was significantly reduced in *tipE*⁻ neurons compared to wild type. Bars indicate SEMs. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, Student's t test.

potential amplitude in the *tipE*⁻ neurons was reduced compared to wild type (Fig. 3D). In contrast, the action potential duration was not different between the two genotypes (Table 2). No significant changes in the input resistance or the resting membrane potential were detected (Table 2). There was a large range in the size of the cultured neurons but the mean capacitance of the population examined in the two genotypes was similar (Table 2).

Rescue of Reduced Repetitive Firing and Spontaneous Firing by Expression of the tipE⁺ Transgene in Differentiated tipE⁻ Neurons

The reduced repetitive firing, spontaneous firing, and action potential amplitude in *tipE*⁻ 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 *Rsa*I restriction enzyme site) generated PCR products of distinct sizes in wild-type and *tipE*⁻ neurons following *Rsa*I digestion (Fig. 4A). In this analysis we found that wild-type and *tipE*⁻ neurons, in both non-heat-shocked and heat-shocked cultures, expressed only wild-type or mutant *tipE* mRNA, respectively. In contrast, *tipE*⁻: *tipE*⁺ neurons express both mutant and wild-type *tipE* 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
Subclass Distribution of Multiple Spiking (MS) Neurons

	Tonic (%MS)	Adaptive (%MS)	Delayed (%MS)
Wild type	53.9 ± 10.8	25.3 ± 10.3	21.3 ± 6.1
tipE⁻	57.4 ± 13.1	20.4 ± 11.7	18.8 ± 13.2

Note. 9 platings; mean ± SEM.

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

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

Note. Mean ± SEM (No. of neurons).

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).

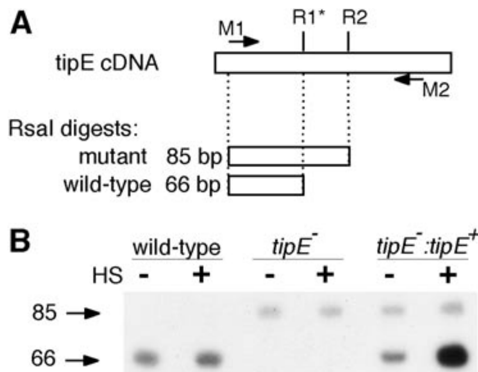

FIG. 4. Heat shock drives expression of wild-type *tipE* mRNA in a *tipE*⁻ background. (A) Schematic representation of the *tipE* cDNA with the orientation of primer pair M1 (radioactively labeled) and M2. Positions of two *Rsa*I restriction enzyme sites, R1* (eliminated in the *tipE* mutant) and R2 are shown. *Rsa*I 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 *Rsa*I 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% CO_2 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

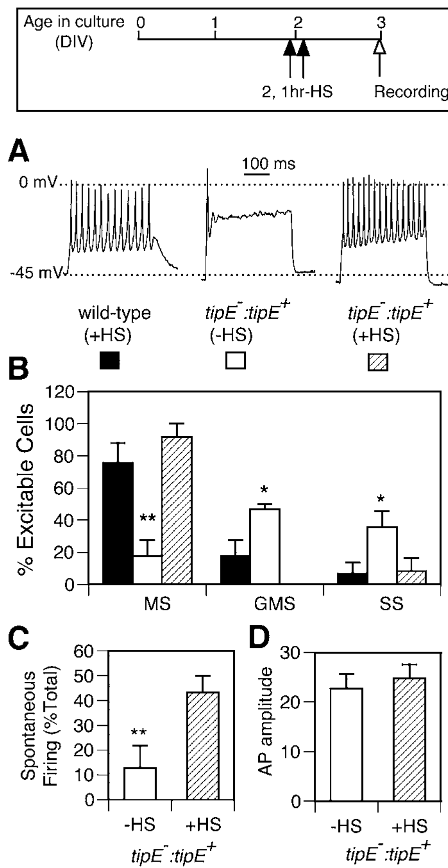

FIG. 5. Expression of wild-type *tipE* message in a *tipE*⁻ background restores wild-type distribution of firing phenotypes and spontaneous firing levels in differentiated neurons. The heat-shock regime used in these experiments is illustrated on the top. Half of the cultures in each genotype were subjected to two 1 h, 37°C heat shocks at 42 and 49 h after plating. Electrophysiological recordings were done at 66–74 h after plating (see Experimental Methods for details). (A) Representative action potential trains recorded from neurons in the three different culture conditions tested, wild-type (+HS), ***tipE*⁻:*tipE*⁺** (−HS), and ***tipE*⁻:*tipE*⁺** (+HS). (B) Heat shock rescued the altered firing class distribution seen in the mutant neurons. The distribution of neurons, normalized to total number of excitable cells, among the three major firing classes was determined in the different cultures. The % multiple-spiking (MS) and % single-spiking (SS) neurons are significantly different in wild type (+HS) and ***tipE*⁻:*tipE*⁺** (+HS) compared to ***tipE*⁻:*tipE*⁺** (−HS) (ANOVA, $*P < 0.05$, $**P < 0.01$, Fisher's protected least significant difference). Heat shock did not alter the distribution of firing classes in wild-type cultures. Mean percentages were determined by calculating the percentages in four independent experiments in which 4–6 neurons/culture condition were examined in each experiment. The total number of neurons in each group was 18 wild type (+HS), 22 ***tipE*⁻:*tipE*⁺** (−HS), and 21 ***tipE*⁻:*tipE*⁺** (+HS). (C) The level of spontaneous firing is restored to wild-type levels in transgenic neurons following heat shock. The fraction of neurons firing spontaneous action potentials, as a function of the total number of neurons examined, is significantly different between ***tipE*⁻:*tipE*⁺** neurons +HS and −HS ($**P < 0.01$, Student's *t* test). (D) The action potential (AP) amplitude is not rescued by induction of the ***tipE*⁺** transgene: there is no significant difference between the mean amplitude in neurons in +HS ($n = 13$) and −HS ($n = 12$) ***tipE*⁻:*tipE*⁺** cultures ($P > 0.05$, Student's *t* test). Bars on all graphs indicate SEM.

heterologous expression system (Feng *et al.,* 1995a; Warmke *et al.,* 1997). In addition, a decrease in sodium current density in ***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 ***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 ***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 ***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

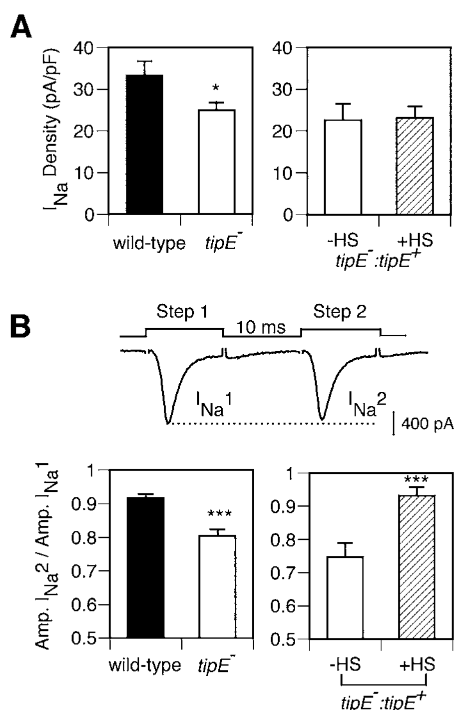

FIG. 6. Sodium currents in wild-type, *tipE⁻*, and transgenic neurons (*tipE⁻:tipE⁺*). (A) Sodium current density is reduced in *tipE⁻* neurons but is not rescued by induction of the *tipE⁺* transgene. Sodium current density was calculated by normalizing the maximal amplitude sodium current to the whole-cell capacitance in each neuron in which a sodium current was observed. There is a significant decrease in mean sodium current density in *tipE⁻* compared to wild-type neurons. $*P < 0.05$, Student's t test, unpaired. The mean sodium current densities in transgenic neurons (*tipE⁻:tipE⁺*) under +HS and −HS conditions are not significantly different from each other ($P > 0.05$, Student's t test). (B) Recovery of sodium currents from repetitive activation is reduced in the mutant and rescued by heat-shock induction of the *tipE⁺* transgene. This was examined using the two-step protocol illustrated; holding potential and recovery voltages were −75 mV, test steps were to −5 mV for 10 ms, interstimulus interval was 10 ms. The sodium current amplitude elicited by the second test step was normalized to that elicited by the first step. The normalized sodium current amplitude in *tipE⁻* neurons ($n = 35$) is significantly lower than in wild type ($n = 29$) ($***P < 0.001$, Student's t test, unpaired). The reduction observed in the transgenic neurons in the absence of heat shock (*tipE⁻:tipE⁺* −HS; $n = 12$) is rescued in the heat-shocked transgenic neurons (*tipE⁻:tipE⁺* +HS; $n = 13$) ($***P < 0.001$, Student's t test, unpaired) ($n = 7$). Bars in all graphs indicate SEM.

normalized to that elicited by the first step. Under these conditions, the amplitude of the second sodium current was approximately 90% of the first sodium current, in wild-type neurons. In contrast, in *tipE⁻* neurons the second sodium current was only 80% of the control amplitude (Fig. 6B). The reduced level of recovery seen 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

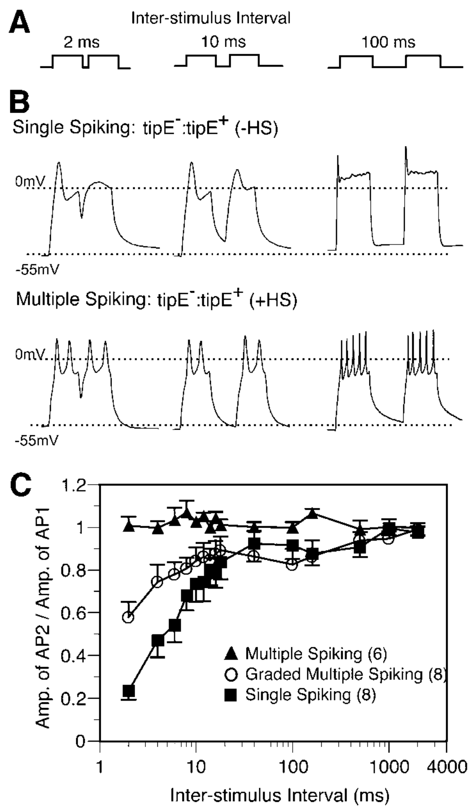

FIG. 7. Recovery of excitability in transgenic neurons. (A) Neurons in transgenic cultures were held at −55 mV and stimulated with the two-step protocols illustrated using three interstimulus intervals (2, 10, 100 ms). (B) Whole-cell current clamp recordings from a single-spiking neuron in the absence 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 in a transgenic culture that 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.

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 than in the multiple-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

tipE in RNA harvested from wild-type cultures between 2 and 48 h *in vitro.* A third primer set was included to amplify ribosomal protein 49 (O'Connell and Rosbash, 1984), serving as a control for normalization of input RNA amounts. The autoradiogram in Fig. 8A illustrates the PCR products generated from total RNA harvested from wild-type neurons at the indicated times in culture. The percentage of the maximal levels of expression, after normalization to rp49 values, is shown in Fig. 8B. Expression of both genes was initiated about 12 h after plating, peaked at 33 h, and subsequently declined to approximately 50–70% of maximum by 48 h (Fig. 8B). The similar time courses of *tipE* and *para* expression are consistent with a role for *tipE* in regulating *para* sodium channels during normal neuronal development.

To determine if *para* and *tipE* are coexpressed in individual neurons, we used a multiplex, single-cell RT-PCR approach to examine their expression in wild-type neurons at 2 days in culture. These analyses were performed with two primer pairs in a single RT-PCR. One primer pair (paraDP3/DP4) flanked the alternatively spliced exons i and a in the *para* gene, amplifying distinct PCR products representing the *para* splice variants containing one or both alternative exons i and a. The second primer set (tipEComF/R) amplified a single PCR product from the *tipE* transcripts. A representative autoradiogram of PCR products amplified from RNA harvested from individual cultured neurons is shown in Fig. 8C. As previously reported there was cell-to-cell variability in expression of *para* splice variants. The majority of cells that express sodium currents and *para* mRNA also express *tipE* (Fig. 8C). In six experiments, in which eight or more neurons were examined/experiment, 92.2 ± 3.1% of the *para*-expressing neurons also expressed *tipE.* These data demonstrate that the *para* and *tipE* gene products could interact directly or indirectly, in single neurons, to influence repetitive firing.

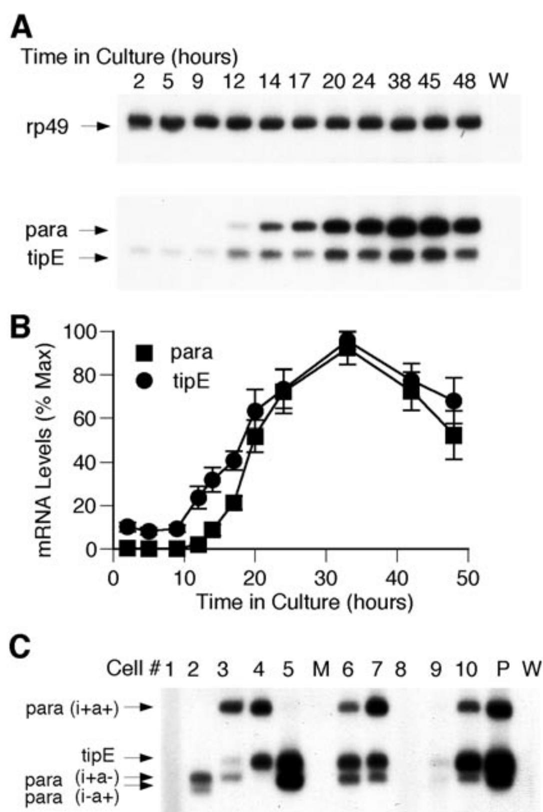

FIG. 8. *tipE* and *para* sodium channel mRNA expressions exhibit similar temporal and spatial distributions in cultured neurons. (A) Autoradiogram of the RT/PCR products separated on an 8% polyacrylamide gel reveals similar temporal patterns of expression of *tipE* and *para* mRNA in the cultured neurons. rp49 is observed at a constant level at all of the ages examined, indicating that expression of this gene is not developmentally regulated and that starting RNA template concentrations were similar in each sample. Lane W represents a negative control in which water is substituted for RNA. PCR products were generated from total RNA prepared from cultured neurons using three individual primer sets that amplify *rp49, para,* and *tipE* transcripts. (B) For temporal comparison the product levels for *tipE* and *para* were normalized to the maximum levels observed for each. Data were obtained from five independent RNA preparations at each age. Bars indicate SEMs. (C) Autoradiogram from a typical experiment showing strong amplification of one or more of three alternatively spliced *para* PCR products from 7/10 cells, with 6/7 of these neurons also expressing the *tipE* product. In addition, low levels of both *para* and *tipE* are seen in one additional neuron (Cell 9). The experiment was accepted for analysis on the basis of clean intermingled medium (M) and water (W) controls that were processed in parallel with the cells. PCR products from total RNA harvested from whole pupae (P) are shown for comparison. Amplification of the cell contents was performed using ^{32}P-labeled primers that amplify the region surrounding alternative *para* exons i and a (paraDP3/DP4) and *tipE* primers that detect all transcripts from the *tipE* region.

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 the mutant *tipE* in cells that fire repetitively. Alternatively, the *tipE* mutant used in this study, an EMS-induced recessive mutation (Kulkarni and Padhye, 1982) resulting in a premature stop codon, may act as a hypomorph 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

TABLE 3
Primer Pairs Used in RT-PCR Studies

Primer name	Sequence	Nucleotide positions	GenBank accession No.
paraComF	TGCCATGTCGTATGACGAATTG	1555–1576	M32078
paraComR	TCTCGCCGCCAACAAATAGC	1796–1777	M32078
paraDP3	ATGTCCATTCGGAGCGTCGA	1826–1845	M32078
paraDP4	CTGGGCATCCTGATATGTTGACA	2083–2061	M32078
tipEComF	AAACTTGAGCAAGACGATGACGAC	2341–2364	U27561
tipEComR	TCTTTTTCGGGTTGGGTCTCC	2545–2525	U27561
tipEM1	TACTATGTGGGAGCCAGGCT	1694–1713	U27561
tipEM2	GAGTAGTAGCAGGGGAACTTCATGC	1815–1791	U27561
rp49F	AAGATGACCATCCGCCCAGCATAC	417–440	X00848
rp49R	CTCGTTCTTCTTGAGACGCAGG	876–855	X00848

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% CO_2 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% CO_2 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 by transfer to a 37°C, 5% CO_2 incubator for 1 h at 42 and 49 h after plating. The remainder of the time they were maintained in a 5% CO_2 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 $\times$ 10^5 dpm of ^{32}P-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 *Rsa*I 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. Phosphoimager 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-

ferentiate between wild-type and *tipE* mutant mRNA, primers tipEM1/M2 were used. *rp49:* rp49F/R primer pair was used for PCR amplification of ribosomal protein transcripts. Sequences, nucleotide positions, and GenBank accession numbers for all of the primer sets used are detailed in Table 3.

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