Novel Compensatory Mechanisms Enable the Mutant KCNT1 Channels to Induce Seizures

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Abstract:

Mutations in the sodium-activated potassium channel (KCNT1) gene are linked to epilepsy. Surprisingly, all KCNT1 mutations examined to date increase K^+ current amplitude. These findings present a major neurophysiological paradox: how do gain-of-function KCNT1 mutations expected to silence neurons cause epilepsy? Here, we use *Drosophila* to show that expressing mutant KCNT1 in GABAergic neurons leads to seizures, consistent with the notion that silencing inhibitory neurons tips the balance towards hyperexcitation. Unexpectedly, mutant KCNT1 expressed in motoneurons also causes seizures. One striking observation is that mutant KCNT1 causes abnormally large and spontaneous EJPs (sEJPs). Our data suggest that these sEJPs result from local depolarization of synaptic terminals due to a reduction in Shaker channel levels and more active Na^+ channels. Hence, we provide the first *in vivo* evidence that both disinhibition of inhibitory neurons and compensatory plasticity in motoneurons can account for the paradoxical effects of gain-of-function mutant KCNT1 in epilepsy.

42 **Introduction**

43

44 Ion channels, such as potassium (K^+), sodium ($Na^{+)}$, and calcium (Ca^{2+})
45 channels, play important roles in neuronal excitability via regulation of the resting
46 potential, the amplitude and the duration of action potentials (APs), the firing rates or
47 patterns of APs, and synaptic transmission. Mutations in voltage-gated cation channels
48 (Na^+, Ca^{2+}, and K^+) that result in neuronal hyper-excitability are often linked to epilepsy[1-
49 3]. One of the distinct exceptions is the sodium-activated potassium channel (KCNT1,
50 also called *Slack or Slo2.2*), which is encoded by the KCNT1 gene and unique in that
51 they are activated by intracellular Na^+ and Cl^-[4-8]. At least 24 point mutations have been
52 found in the human *KCNT1* gene in patients with autosomal dominant nocturnal frontal
53 lobe epilepsy (ADNFLE), malignant migrating partial seizures of infancy (MMIPSI), early
54 onset epileptic encephalopathy (EOEE), and West syndrome[9-15]. The number of
55 patients affected by KCNT1 mutations is not known but many of these patients do not
56 respond well to current anti-epilepsy drugs[13,16-18].

57 At present, the cellular mechanisms of KCNT1-related epilepsy remain poorly
58 understood. Electrophysiological studies using heterologous expression systems such
59 as the *Xenopus* oocytes or Chinese hamster ovary (CHO) cells have shown that all of
60 the mutant KCNT1 channels studied to date significantly increase the magnitude of K^+
61 currents[9,11,12,19]. These findings are important but they present an interesting
62 neurophysiological conundrum in that increased K^+ currents are expected to
63 hyperpolarize or silence neurons and reduce the possibility for neurons to fire APs, a
64 condition paradoxically unfavorable of triggering seizure or epilepsy.

65 Two different hypotheses have been proposed to explain this puzzle. The
66 'repolarization hypothesis'[5,20-23] states that increased Na^+-dependent K^+ currents might
67 accelerate the rate of AP repolarization, thus enhancing the firing rate. Studies of
68 KCNT1 KO mice showed, however, that the lack of the KCNT1 K^+ current sped up
69 repolarization and enhanced AP firing[24]. This result is not in agreement with the
70 'repolarization hypothesis', suggesting that KCNT1 K^+ current hinders repetitive firing
71 normally. Another hypothesis is the 'disinhibition hypothesis'[19], which states that
72 increased K^+ currents in inhibitory interneurons reduce GABA release and consequently
73 cause hyperexcitability of postsynaptic neurons. This hypothesis is plausible; however,
74 it has not been tested directly.
75 To test the disinhibition hypothesis and to discover new *in vivo* function of mutant
76 KCNT1, we generated a fruit fly model of human mutant KCNT1. Our data show that
77 expression of the mutant KCNT1 channels in GABAergic interneurons indeed causes
78 bang-sensitive seizures in flies, the first *in vivo* observation in support of the disinhibition
79 hypothesis. To our surprise, however, expression of the mutant channels in
80 motoneurons results in uncoordinated larval locomotion, enhanced synaptic
81 transmission, and also bang-sensitive seizures in adult flies. These results suggest that
82 neuronal excitability is enhanced rather than reduced in motoneurons by the mutant
83 KCNT1. We further show that neuronal hyperexcitability is achieved in part by a
84 compensatory downregulation of endogenous *Drosophila* K^+ channels such as Shaker
85 and enhancement of voltage-gated Na^+ channels, in an attempt to counter balance the
86 effect of increased KCNT1 K^+ current. Hence, our study supports two complementary
87 hypotheses, the 'disinhibition hypothesis' and a new 'compensatory plasticity

88 hypothesis', to help better understand the neurobiology underlying KCNT1-associated
89 epilepsy.
90
91 **Results**
92 **Mutant hKCNT1 channels increase K^+ conductance in muscles**
93 To date, nearly all electrophysiological studies of mutant KCNT1 channels
94 associated with epilepsy have been conducted in heterologous expression systems,
95 such as the Xenopus oocytes or CHO cells[9,11,12,19]. These studies show that mutant
96 hKCNT1 channels display a significant increase in K^+ current magnitude compared to
97 control[9,11,12,19]. To determine whether hKCNT1 mutations also exhibit gain-of-function
98 (an increase in K^+ conductance) phenotypes *in vivo*, we used the *GAL4/UAS* system to
99 overexpress wild type (WT) human KCNT1 (hKCNT1) and two mutants, G288S (a point
100 mutation in S5; next to the channel pore) and R928C (a point mutation near the NAD^+
101 domain) (Suppl Fig. 1a). Our data show that the resting potential is significantly
102 hyperpolarized and the input resistance dramatically reduced in muscles expressing
103 either G288S or R928C *hKCNT1* mutations by *Mhc-GAL4* (Suppl Fig. 1b-e). The
104 *hKCNT1* mutant larvae were not able to crawl very far from its starting point and for the
105 most part of the recording period the larvae were stationary and inactive (Suppl Movie 1
106 and Suppl Fig. 2). These results are similar to the effects of human inward-rectifier
107 potassium channel 2.1 (*Kir2.1*)[25] expressed in muscles (Suppl Fig. 1b-e). Thus, our *in*
108 *vivo* data illustrate that *hKCNT1* G288S and R928C mutations are gain-of-function
109 resulting in an increase in K^+ conductance, and subsequently leading to impaired larvae
110 locomotion activities, hyperpolarized resting potential, and lower muscle resistance.

111 **Mutant KCNT1 causes seizures in adult flies**

112 Epilepsy is neurological disease defined as involuntary muscle convulsions
113 resulting in seizures. Drosophilists have been using adult flies to determine the genetic
114 bases of epilepsy and whether a particular mutation will predispose the flies to seizures.
115 Several behavior and electrophysiological techniques have been developed to assess
116 seizures in adult flies[26-28]. The bang-sensitivity behavioral assay is used to determine
117 whether subjecting flies to mechanical stimulation can elicit seizure activities[29,30].

118 To directly test the disinhibition hypothesis, we expressed the mutant and
119 wildtype human KCNT1 in GABAergic neurons using a cell-specific *Gal4*, *Gad-Gal4*[31].
120 Adult flies were then subjected to the bang-sensitive test in which the flies were placed
121 in an empty fly vial, vortexed for 20 sec, and then observed for seizing behavior (often
122 flip on their backs and unable to stand up or buzz around for at least one second). We
123 noted that the flies expressing mutant R928C in GABAergic neurons had a higher
124 propensity to display seizure (Fig. 1a-e; Suppl Movie 2). The frequency of seizures is
125 significantly higher and duration significantly longer compared to the two control groups
126 (*Gad-Gal4 >WT*; *Gad-Gal4 > WT human KCNT1*). The mean seizing frequency is 26%
127 compared to the controls (10%, 5%, respectively). The mean seizing duration of these
128 flies is significantly longer ($p<0.01$), averaging 28 Sec compared to 2 sec and 1.5 sec in
129 *Gad-Gal4>WT* and *Gad-Gal4>WT human KCNT1*, respectively. These results support
130 the 'disinhibition hypothesis', indicating that silencing inhibitory neurons tips the balance
131 towards excitation and thus implicating a role for GABAergic neurons in epilepitogenesis
132 in human patients.

133 We were also curious about whether expression of the mutant KCNT1 channels
134 in motoneurons would have a similar effect as they did in GABAergic neurons. In
135 motoneurons mutant KCNT1 driven by *D42-Gal4*[32,33] causes embryonic lethality at 25
136 °C, consistent with the silencing effect of enhanced K^+ current and pan neuronal
137 expression of D42 in early embryos. To overcome this obstacle, we used *GAL80*^{ts} [34]
138 (this form of *GAL80* become inactive when flies are reared at higher temperature) in the
139 context of the *D42, UAS hKCNT1* mutation to prevent the expression of hKCNT1
140 channels during development. Flies were kept at 18 °C until eclosion, then adult flies
141 were collected and kept at 25 °C for two weeks, allowing the expression of hKCNT1
142 mutant channels in adult flies. Mechanical stimulation of flies expressing R928C
143 mutation by vortexing showed a significant increase in the percentage of seizing flies
144 and seizing duration (25.7 ± 3.5 S and 11.3 ± 2.1 S, respectively) compared to *D42/+*
145 (1.25 ± 0.9 S and 1 S, respectively) and *D42/hKCNT1* (0.7 ± 0.7 S and 1 S,
146 respectively, Fig. 1f-j and Suppl Movie 3). These data suggest a new mode by which
147 mutant hKCNT1 channels cause seizure and epilepsy.
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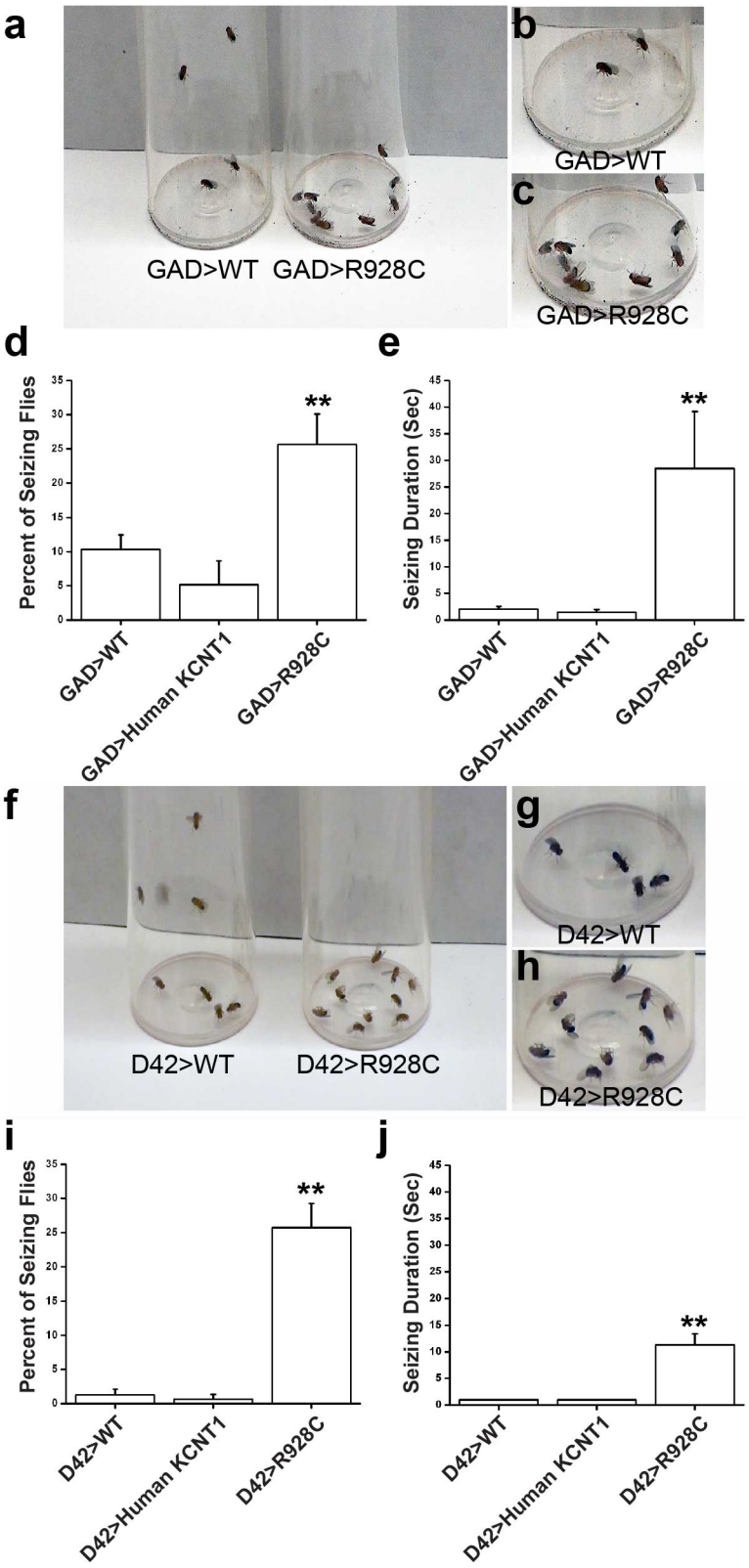
a
GAD>WT
GAD>R928C
b
GAD>WT
c
GAD>R928C
d
Percent of Seizing Flies
**
GAD>WT
GAD>Human KCNT1
GAD>R928C
e
Seizing Duration (Sec)
**
GAD>WT
GAD>Human KCNT1
GAD>R928C
f
D42>WT
D42>R928C
g
D42>WT
h
D42>R928C
i
Percent of Seizing Flies
**
D42>WT
D42>Human KCNT1
D42>R928C
j
Seizing Duration (Sec)
**
D42>WT
D42>Human KCNT1
D42>R928C

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Figure 1: Neuronal expression of mutant hKCNT1 channel leads to seizures in adult flies.
(**a**, **f**) Representative image of flies after twenty seconds of mechanical stimulation (vortexing). Close-up images of the bottom of the vials are shown in (**b**, **c**, **g**, and **h**). Control flies show no sign of seizure activity after vortexing whereas a large number of mutant KCNT1-expressing flies exhibit seizure. (**d** and **e**) GABAergic expression of mutant hKCNT1 channel in adult flies show a significant increase in both number of seizing flies and seizing duration. (**i** and **j**) Motoneuron expression of mutant hKCNT1 channel predispose flies to seizure and very few control flies seized after vortex. If control flies seized they did not seize more than one second while flies expressing mutant hKCNT1 channels showed significantly longer seizure durations. Flies were reared at 18 °C until eclosion and adult flies were reared at 25 °C from 10-15 days before testing. At least 120 and 40 flies were tested for Motoneuron and GABAergic expression per genotype. Error bars show S.E.M. $^{**}P < 0.01$ one-way ANOVA, Turkey HSD *post hoc test*.

152

153 **Mutant hKCNT1 channels do not silence motoneurons but disrupt coordinated**

154 **crawling and motoneuronal firing**

155 The findings from expressing mutant KCNT1 in muscle, GABAergic neurons, and

156 motoneurons appear to contradict one another in that muscles and GABAergic neurons

157 are silenced whereas motoneurons in adult flies are not. In order to better understand

158 the mechanisms by which mutant KCNT1 in motoneurons causes seizures, we decided

159 to examine the impact of mutant KCNT1 on larval behavior and physiology. To

160 overcome the embryonic lethality, we raised the flies with motoneuronal expression of

161 mutant KCNT1 at 18°C, which reduces the expression levels of the mutant hKCNT1

162 channels. *D42>G288S* reached pupal stage and few of the *D42>R928C* flies were able

163 to reach the adult stage. We noted that larvae expressing the mutant KCNT1 channels

164 showed severe crawling defects compared to control larvae (wildtype or larvae

165 expressing the wildtype KCNT1) (Suppl Fig. 3a,b; Suppl Movie 4). Because of the

166 uncoordinated crawling, the pupae became bended (crescent shape, Suppl Fig. 3c).
167 However, after careful observation we noted that unlike muscle expression of mutant
168 hKCNT1 channels, which were inactive for the most part of the observation, larvae
169 expressing G288S or R928C in motor neurons showed a strong and active but one
170 sided larval contractions (Suppl Movie 4). This finding suggests that the reduction in
171 larvae crawling distance is not due to neuronal inactivity but rather uncoordinated
172 neuronal firing.

173 Our behavioral observations of both larvae and adults strongly suggest that
174 neuronal expression of mutant KCNT1 at low levels does not silence motoneurons. On
175 the contrary, they cause hyperactivity in larvae (see below) and seizures in adult. To
176 determine the effects of *hKCNT1* mutations on neuronal activity, we utilized the well-
177 established neuromuscular junction (NMJ) preparation to assess their effects on
178 synaptic transmission in third-instar larvae[35,36]. Surprisingly, electrical stimulation of the
179 segmental nerve did not alter the amplitude of excitatory junction potentials (EJPs) of
180 larvae expressing G288S or R928C mutations in motor neurons compared to control
181 larvae. The mean EJP amplitude for *D42-GAL4>G288S*, *D42>R928C* was 49.1 ± 1.7
182 mV and 49.5 ± 1.7 mV, respectively, which were not significantly different from *D42/+*
183 (50.6 ± 1.1 mV) and *D42/hKCNT1* (49.5 ± 1.3 mV) (Fig. 2a,b). In addition, there was no
184 significant difference in membrane resting potentials between of G288S and R928C
185 mutations and controls (*D42/+* and *D42/hKCNT1*) (Suppl Fig. 4). These data indicate
186 that reduction of larval crawling activity caused by motoneuronal expression of mutant
187 hKCNT1 channels is not due to neuronal silencing but most likely due to inability to
188 coordinate neuronal firing, which hinders larvae crawling ability.

189 To more directly investigate whether *hKCNT1* mutations compromise
190 coordinated neuronal firing, we examined ventral nerve cord (VNC) neuronal firing in
191 third-instar larvae using Ca^{2+} imaging. Larvae VNC (equivalent to spinal cord in
192 humans) have eight pairs of abdominal ganglia, each right and left ganglion controls the
193 corresponding larval body wall segment[37-40]. For example, the first pair of ganglia
194 control the right and left abdominal muscle segment 1. During larvae crawling the first
195 pair of ganglia will fire together then the next pair and signal will propagate down until
196 the last pair fired[37-40]. To visualize ganglia firing we used the calcium sensor *GCaMP6S*
197 expressed in motoneurons. Larvae expressing WT hKCNT1 channels showed
198 coordinated ganglion firing, where right and left ganglia fired simultaneously (Fig 2c,d
199 and Suppl Movie 5). On the other hand, expression of hKCNT1 mutated channels
200 resulted in a significant number of unsynchronized motoneuronal firing, and sometimes
201 only ganglion in one side fired without the other pair firing at all (Fig. 2c,d,e and Suppl
202 Movie 5). In addition, oftentimes calcium waves were not able to pass through all
203 ganglia and usually terminated after reaching the fourth ganglion in larvae expressing
204 mutant hKCNT1 channels. However, both larvae expressing WT and mutated hKCNT1
205 channels showed a similar peak amplitude of Ca^{2+} waves (Fig. 2f). Taken together, our
206 data on both EJP and calcium waves suggest that hKCNT1 mutations do not silence
207 neuronal firing but rather influence firing synchrony.
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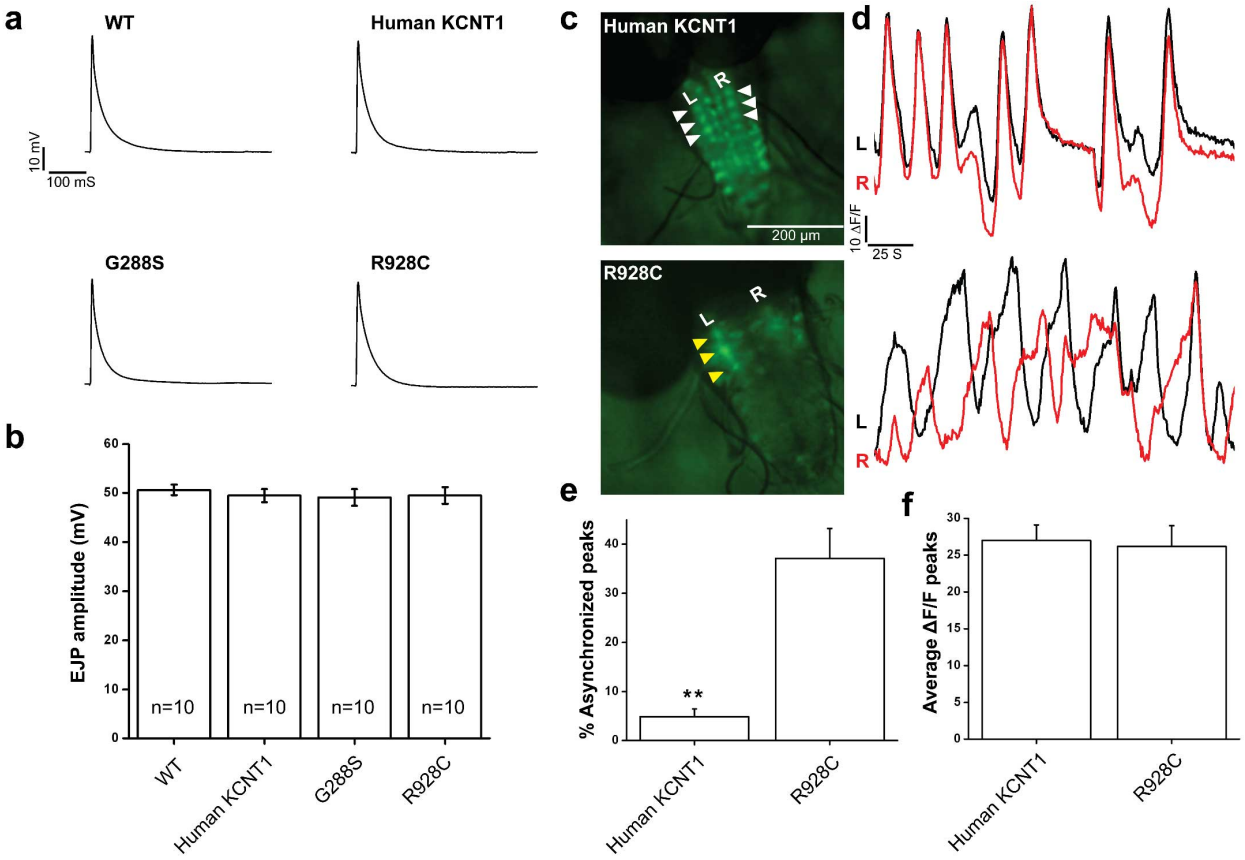

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Figure 2: Expression of *hKCNT1* mutation did not silence neurons, but caused uncoordinated ventral ganglion firing.
(**a**) Representative traces of excitatory junction potential (EJPs) under physiological conditions. (**b**) Average EJPs amplitude of ten recordings (two per larva) per genotype. Recordings were taken from muscle 6 segment A3 or A4. (**c**) Representative image of calcium waves in larvae expressing WT hKCNT1 channels (top) in motor neurons and larvae expressing mutated hKCNT1 channel (bottom). The calcium waves were visualized using *GCaMP6S* calcium sensor expressed in motoneuron. **L** and **R** indicate left and right sides, respectively, of ventral nerve cord (VNC) in third-instar larvae. White and yellow arrow heads indicate coordinated and uncoordinated left and right VNC ganglion firing respectively. (**d**) Representative calcium waves signals from left and right VNC ganglion pairs, showing coordinated (top) and uncoordinated (bottom) waves in larvae expressing WT and mutated hKCNT1 channels during active crawling respectively. (**e**) Quantification of percent of uncoordinated left and right VNC ganglion calcium waves. (**f**) Quantification of average calcium wave amplitude. Five larvae were used for calcium waves analysis. All larvae were reared at 22 °C for 5 days and 2 days at 25 °C prior to EJP recordings and calcium imaging. Error bars show S.E.M. $^{**}P < 0.01$, one-way ANOVA, *Student's T test*.

211

212 **Mutant hKCNT1 channels cause large spontaneous postsynaptic potentials at the**
213 **larval NMJ**

214 Spontaneous miniature excitatory junction potentials (mEJPs or minis) play a role
215 in synaptic plasticity and function, evoked transmitter release, neuronal excitability, and
216 postsynaptic membrane resistance[41-45]. It has been shown that in somatostatin cells
217 taken from epileptic mice exhibit an increase in miniature excitatory postsynaptic
218 synaptic current (mEPSC) frequency[46]. Thus, we investigated whether *hKCNT1*
219 mutations alter the mini properties at the NMJ of third-instar larvae. We severed the
220 segmental nerves posterior to the ventral ganglion and monitored mEJP activity (usually
221 spontaneous release from single vesicles) at the NMJ.

222 Control larvae (both *D42/+* and *D42/hKCNT1*) displayed typical spontaneous
223 minis, with an average frequency of 1.4 Hz and amplitude of 1.7 ± 0.1 mV (for both
224 genotypes). Remarkably, larvae expressing mutant hKCNT1 channels showed very
225 large spontaneous synaptic potentials, up to 14 mV (Fig. 3a). As shown below, these
226 large synaptic events are spontaneous EJPs. Hence, we will refer them as spontaneous
227 EJPs (sEJPs) rather than minis or mEJPs. The average amplitude of spontaneous
228 synaptic potentials (note that this refers to both minis and sEJPs) in larvae expressing
229 G288S and R928C mutations was significantly higher (2.6 ± 0.2 mV and 3 ± 0.2 mV,
230 respectively, ANOVA, $P < 0.01$). The counts of minis plus sEJPs and their cumulative
231 probability plots are shifted to the right in larvae expressing mutant hKCNT1 channels,
232 due to the presence of significantly high number of large sEJPs in these larvae
233 compared to *D42/+* and *D42/hKCNT1* larvae (Fig. 3b,c).

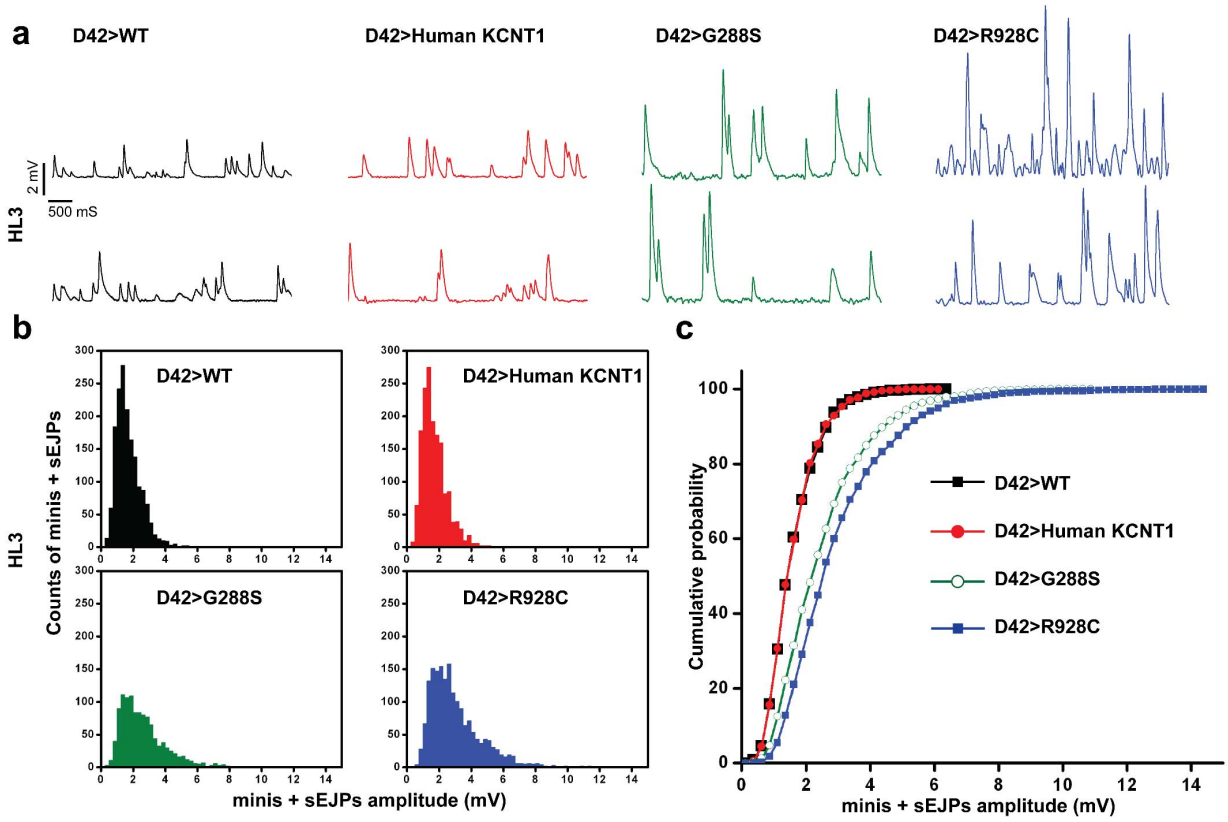

Figure 3: Third-instar larvae expressing *hKCNT1* mutations in motor neurons showed spontaneous EJPs.
(**a**) Representative traces of miniature excitatory junction potentials (mEJP or minis) under physiological condition. Both *hKCNT1* mutants show significantly unusually larger spontaneous synaptic events in addition to minis compare to *D42/+* and *D42/hKCNT1* traces. We call these large synaptic potentials spontaneous EJPs (sEJPs). (**b**) Histograms of spontaneous synaptic potentials (minis plus sEJPs) of the four different genotypes. Synaptic potential counts are sorted into 0.125 mV bins. Note the right-shift distribution of spontaneous synaptic potentials in the two mutant KCNT1 larvae. (**c**) Cumulative probability plot of spontaneous synaptic potentials from larvae expressing mutant hKCNT1 channels illustrate the present of significantly higher number of large sEJPs compare to *D42/+* and *D42/hKCNT1*. Recordings were taken from muscle 6 segment A3 or A4 and all larvae were reared at 22 °C for 5 days and 2 days at 25 °C prior to synaptic recordings. A total of ten recordings (two per larva) were made per genotype.

These sEJPs are highly unusual as the axons of the motoneurons are cut free from the soma and it is not possible for action potentials to be propagated from the

238 soma. We reasoned that they were caused by local depolarization at the synaptic
239 terminal. This could be possible only if there was a compensatory enhancement in
240 voltage-gated cation (Na^+ or Ca^{2+}) channel levels and activities or a reduction in K^+
241 channel levels and activities. To test this hypothesis, we used tetrodotoxin (TTX) to
242 block voltage-gated sodium channel activity and surprisingly observed that TTX only
243 reduced the number of 'extremely' large sEJPs (>10 mV) but did not abolish sEJPs in
244 larvae expressing mutant hKCNT1 channels (Fig. 4a,b,c; Suppl Fig. 5). However, we
245 noticed a slight decrease in average amplitude of spontaneous synaptic potentials in the
246 G288S and R928C expressing larvae (2.1 ± 0.1 mV and 2.5 ± 0.2 mV, respectively,
247 ANOVA, $P < 0.01$), but TTX had no significant effect on mini amplitude in *D42/+* and
248 *D42/hKCNT1* larvae (1.6 ± 0.1 mV and 1.5 ± 0.1 mV, respectively). These data suggest
249 that there is an upregulation of voltage-gated Na^+ channels but they are not responsible
250 for eliciting the sEJPs.
251 We then added the Ca^{2+} channel blocker cadmium to the TTX-containing saline
252 and effectively eliminated the sEJP in the mutant KCNT1-expressing larvae (Fig. 4d,e,f).
253 On the other hand, no changes in mEJP properties were observed in the *D42/+* and
254 *D42/hKCNT1* larvae. These results indicate that depolarization of voltage-gated Ca^{2+}
255 channels at the NMJ likely results in more transmitter release and gives rise to sEJPs.
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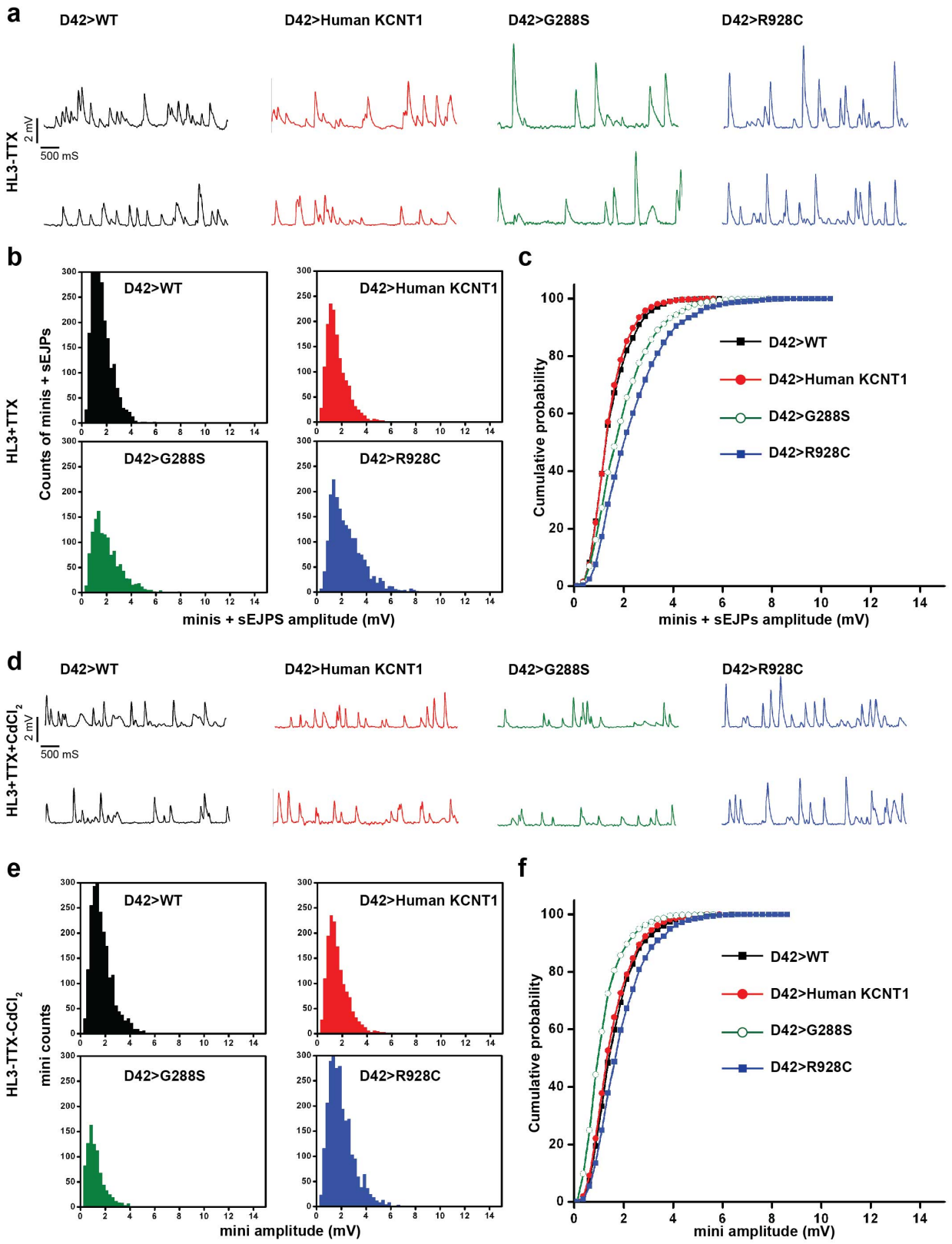

a
D42>WT
D42>Human KCNT1
D42>G288S
D42>R928C
HL3-TTX
2 mV
500 mS
b
HL3+TTX
Counts of minis + sEJPs
D42>WT
D42>Human KCNT1
D42>G288S
D42>R928C
minis + sEJPS amplitude (mV)
c
Cumulative probability
D42>WT
D42>Human KCNT1
D42>G288S
D42>R928C
minis + sEJPs amplitude (mV)
d
HL3+TTX+CdCl2
2 mV
500 mS
e
HL3-TTX-CdCl2
mini counts
mini amplitude (mV)
f
Cumulative probability
mini amplitude (mV)

258

259

Figure 4: Blocking both voltage-gated sodium and calcium channels in larvae expressing mutant hKCNT1 channels eliminated the spontaneous EJPs. (**a**) Representative traces of spontaneous synaptic potentials (minis + sEJPs) treated with voltage-gated sodium channel blocker TTX (1 μM) in HL-3 saline. Note that the large sEJPs persist in TTX-saline. (**b** and **c**) Histograms and cumulative probability plots show that the extremely large sEJPS (>10 mV) are blocked by TTX but TTX does not eliminate all sEJPs. (**d**) Representative traces of spontaneous synaptic potentials (minis + sEJPs) treated with both voltage-gated Na^+ channel blocker TTX (1 μM) and voltage-gated K^+ channel blocker $CdCl_2$ (10 μM) in HL-3 saline (This treatment successfully reduces or abolishes the occurrence of spontaneous EJPs. (**e** and **f**) Histograms and cumulative probability plots show similar distributions of spontaneous synaptic potentials in TTX and Cd^{2+}-treated larval NMJs. Recordings were taken from muscle 6 segment A3 or A4 and all larvae were reared at 22 °C for 5 days and 2 days at 25 °C prior to mini recordings. A total of ten recordings (two per larva) were made per genotype.

260

261 **Immunocytochemistry reveals compensatory decreases in endogenous K^+**

262 **channels in larvae expressing mutant KCNT1**

263 Our behavioral, imaging, and electrophysiological studies collectively suggest

264 that mutant KCNT1 channels trigger compensatory mechanisms by which other ion

265 channels are down or up regulated to counter balance the silencing effect of KCNT1 K^+

266 channels. In other words, the large increase in K^+ currents through mutant KCNT1

267 channels has the potential to fully or partially silence neurons, like Kir2.1 does, to

268 reduce the possibility of firing and impair synaptic transmission. In contrast, the

269 motoneurons appear to fire normally (albeit not synchronized) and the EJP amplitude is

270 largely unchanged. What enables the motoneurons to fire action potentials and the

271 synaptic terminal to produce local and spontaneous auto-depolarization? We

272 hypothesize that there is a compensatory change of excitability at both cell bodies and

273 axons to ensure that action potentials can be produced and propagated. Furthermore,

274 we hypothesize that there is a local compensation at the NMJ to enhance synaptic

activity. We used antibodies specific to Shaker (Sh) to stain the CNS and the neuromuscular preparation of larvae and showed that Shaker channels are reduced in levels (Fig. 5a,b).

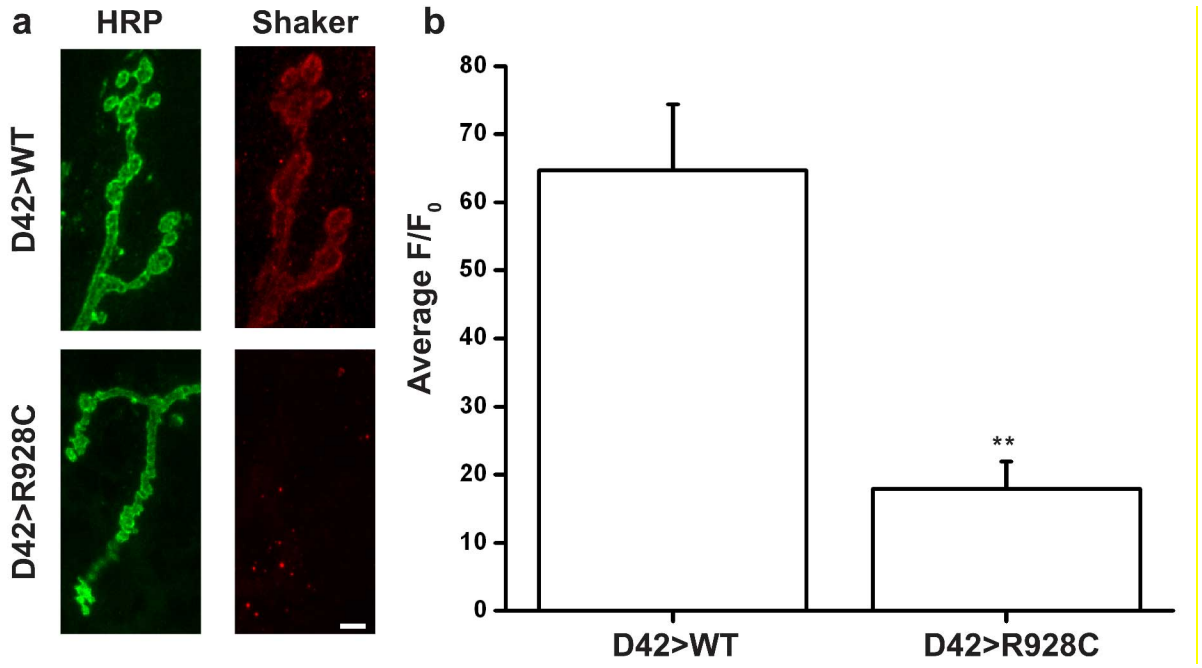


Figure 5: Neuronal expression of mutant hKCNT1 channel leads to significant reduction in Shaker channels staining at the NMJ.
(**a**) Representative NMJ confocal images of WT and larvae expressing mutant hKCNT1 channel in motoneurons. Larvae expressing mutant hKCNT1 channel show weak or no Shaker staining. Scale = 5 μM. (**b**) Quantification of Shaker staining intensity show that Larvae expressing mutant hKCNT1 channel have a significant reduction in Shaker signal compared to control. $^{**}P < 0.01$, *Student's T test*.

Discussion

The discovery of the link between mutant KCNT1 and epilepsy is important but it also presents challenges understanding the role of the K^+ channel in epileptogenesis. *In vitro* studies to date all show that mutant KCNT1 channels significantly increases K^+

286 current magnitude[9,11,12,19]. It is easier to understand why mutations enhancing Na^+ and
287 Ca^{2+} channel activities or reducing K^+ channel activities can cause hyperexcitability and
288 increase the probability for seizures or epilepsy. Similarly, mutant Cl^- channels in
289 skeletal muscles both in the fainting goat and humans cause myotonia[47-49].
290 Neurophysiologically speaking, enhancing K^+ currents is expected to hyperpolarize the
291 resting potential or truncate action potentials and thereby reducing the possibility for
292 neuronal firing. What then accounts for the neuropathology in KCNT1-associated
293 epilepsy?
294 Two different hypotheses have been proposed to explain why enhanced K^+
295 channel activities in mutant KCNT1 could cause hyperexcitability and generate
296 conditions in favor of seizures. One hypothesis, which we name 'repolarization
297 hypothesis', states that following Na^+ influx the activation of KCNT1 K^+ channels
298 shortens the duration of APs by repolarizing it at a faster rate. This in turn enables the
299 neuron to fire more APs per unit time, resulting in hyperexcitability. This possibility is
300 plausible, as shown in electrocytes of some electrical fish[20] and BK channel and β
301 subunit -linked epilepsy[50-52]. However, it will depend on the mode by which Na^+
302 activates KCNT1 channels and the kinetics of the $K_{(Na)}$ current[53]. KCNT1 channels are
303 unique in that is activated by intracellular Na^+ and Cl^-. Salkof and colleagues showed
304 that activation of KCNT1 channels does not need high intracellular $[Na^+]_i$[54], implying that
305 KCNT1 may also be important for contributing to resting potential as well as repolarizing
306 AP. Furthermore, $K_{(Na)}$ following Na^+ influx could outlast the duration of an AP,
307 especially during afterhyperpolarization period, and therefore prevent or delay the onset
308 of the next AP. Finally, genetic studies of KCNT1 KO in mice showed that AP

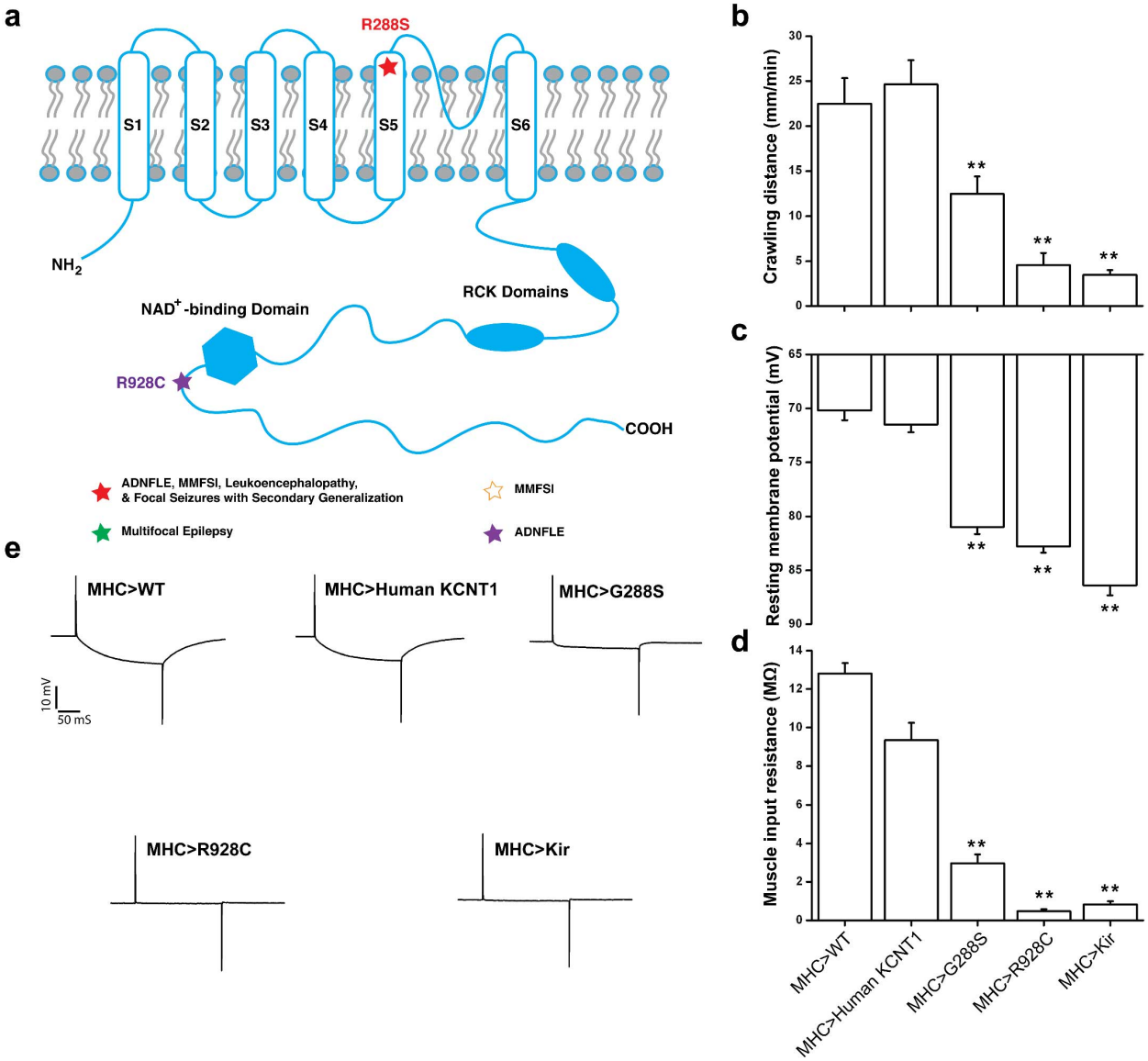
309 repolarization is faster and neurons fire more APs[24]. This *in vivo* study indicates that the
310 presence of KCNT1 K current normally hinders excitability or silence neurons. Our data
311 obtained from muscle expression of the mutant KCNT1 provide strong evidence that the
312 mutant KCNT1 channels are similar to inward rectifier K^+ channel 2.1 in reducing the
313 muscle input resistance and hyperpolarizing the muscle resting potential. Further,
314 neuronal expression of the mutant KCNT1 at high levels causes embryonic lethality or
315 folded wings (Suppl Fig. 6) if the flies live to adult. These observations are consistent
316 with the notion that mutant KCNT1 are gain of function mutations that silence both
317 muscles and neurons. Hence, studies in both mice and fruit flies do not support the
318 'repolarization hypothesis'.

319 The second hypothesis, which we call the 'disinhibition hypothesis'[19], postulates
320 that the enhanced mutant KCNT1 K^+ current silences inhibitory neurons and thereby
321 removing inhibition of neural circuits and tipping the balance towards hyperexcitability
322 and seizures. This is an exciting hypothesis, but up to this point this hypothesis has not
323 been directly tested. Our data showing that expression of mutant KCNT1 in GABAergic
324 neurons induces seizures in adult flies lends a strong support to this hypothesis.

325 However, the disinhibition hypothesis alone may not be sufficient to account for
326 the mutant KCNT1 actions in the nervous system. This is because KCNT1 is broadly
327 expressed in a variety of neuronal types and regions in the human brain[55,56]. The broad
328 and complex expression pattern of KCNT1 begs for additional mechanisms other than
329 the disinhibition hypothesis to account for the effect of mutant KCNT1 in other neurons.
330 By expressing the mutant KCNT1 in motoneurons we have learned that these neurons
331 can be silenced if the expression levels are high or become hyperexcited if the

332 expression levels are low. When specifically expressed in adult motoneurons, mutant
333 KCNT1 also causes bang-induced seizures, suggesting that these neurons are not
334 silenced. This is consistent with the observations of normal EJPs and Ca^{2+} peaks in
335 motoneurons in larvae. More strikingly, we reveal novel changes at the synapse where
336 local depolarization leads to spontaneous synaptic potentials, which is partially sensitive
337 to TTX blockade and fully sensitive to Cd^{+}. At the NMJ, the major endogenous K^{+}
338 channel Shaker is significant reduced in levels, and TTX can block some of the sEJPs,
339 providing a molecular explanation for the enhanced excitability in motoneurons. Our
340 finding of sEJPs is consistent with previous findings that sEJPs also occurred in flies
341 with mutations in Sh eag K^{+} channels[57]. Based on these observations, we propose a
342 'compensatory plasticity hypothesis' as a novel mechanism to counter balance the
343 silencing effect of mutant KCNT1 currents as an additional means to produce neuronal
344 hyperexcitability and seizures. This work adds to the growing list of examples of
345 homeostatic regulation of neuronal excitability[58], although in our KCNT1 fly model it fails
346 to fully compensate to restore normal function. The disinhibition and compensatory
347 mechanisms are complementary to each other, suggesting that mutant KCNT1
348 channels likely have different effects on different neurons. Further studies are expected
349 to pinpoint the regulatory mechanisms by which mutant KCNT1 regulates Sh, Na^{+} and
350 perhaps ion channel gene expression or trafficking.
351
352
353
354

355 **Supplementary Figure legends**



356

357 **Supplementary Figure 1: Muscle expression of mutant hKCNT1 protein in third-**

358 **instar larvae reduce crawling distance, membrane resting potential, and muscle**

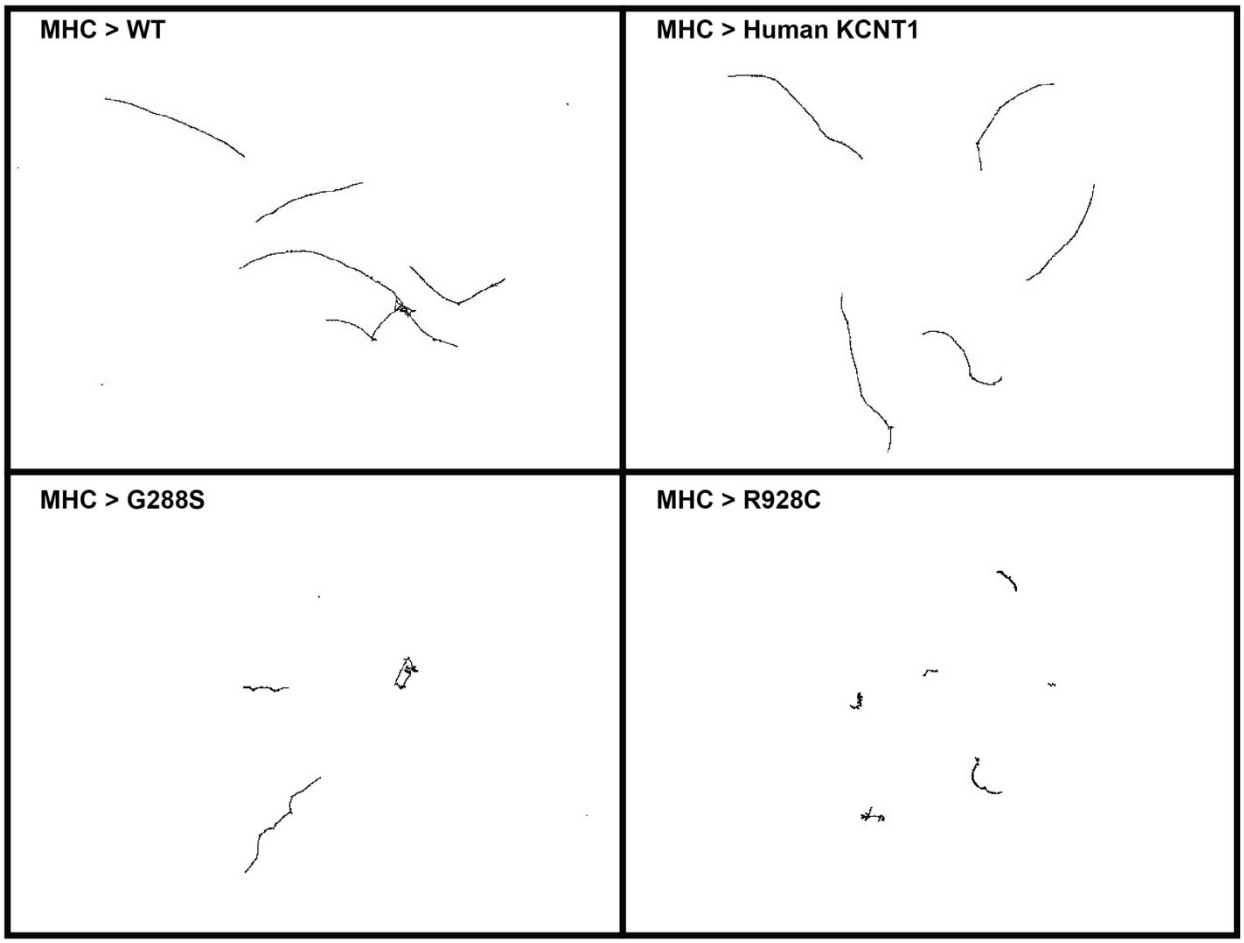
359 **resistance.**

360 (**a**) Schematic diagram of human KCNT1 protein showing protein structure, location of

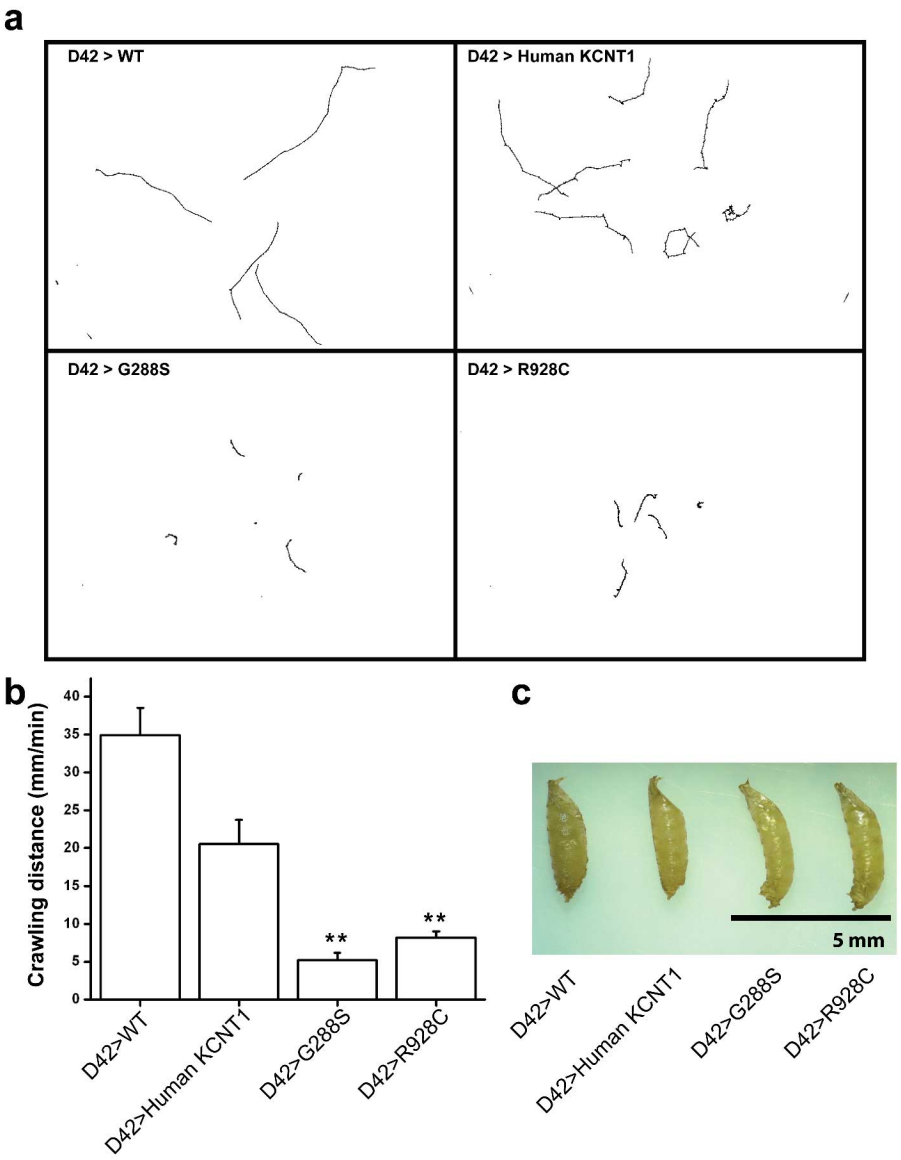
361 the mutations studied in this paper, and type of epilepsy syndrome caused by these

362 mutations. (**b**) Third-instar larvae expressing either mutant *hKCNT1* or *Kir2.1* in muscles

363 show a significant reduction in average crawling distance compare to controls. At least
364 twenty larvae were used per genotype (10 males and 10 females). (**c** and **d**) In addition,
365 overexpression of mutant KCNT1 channels significantly hyperpolarized the average
366 resting membrane potential (**c**) and average muscle input resistance (**d**). (**e**)
367 Representative traces for muscle input resistance in control and experimental larvae.
368 Note the dramatic and similar reduction in muscle input resistance in both *MHC>R982C*
369 and *MHC>Kir2.1* larvae. Muscle 6 of abdominal segment 3 or 4 was used for assisting
370 the resting membrane potential and muscle input resistance. Ten larvae were used per
371 genotype. All larvae were kept at 25 °C until the time of testing. Error bars show S.E.M.
372 $^{**}P < 0.01$, one-way ANOVA, Turkey HSD *post hoc test*.
373



Supplementary Figure 2: Representative larvae crawling tracks. Larvae expressing mutant hKCNT1 channels in muscles show significant reduction in crawling distance compared to controls.



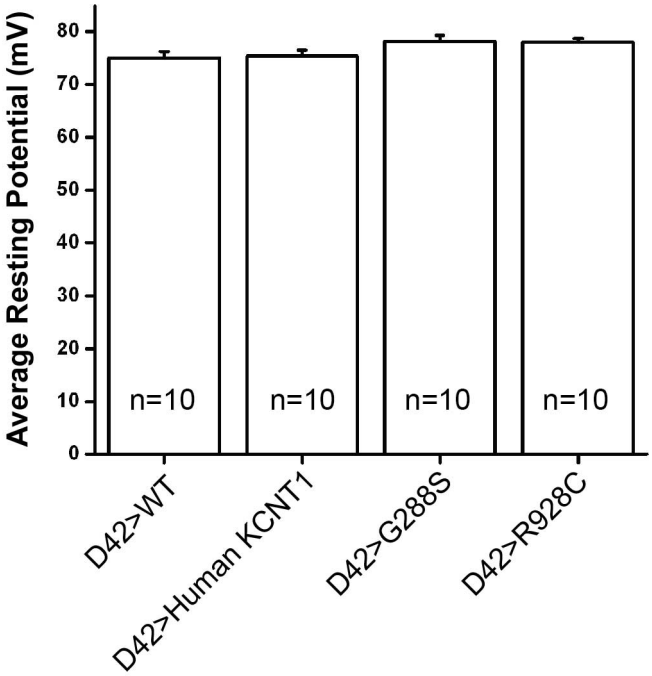
Supplementary Figure 3: Motor neuronal expression of mutant hKCNT1 channels alter pupal and adult wings morphology and reduces third-instar larval crawling activity.

(**a**) Representative larvae crawling tracks. (**b**). (**c**) Motor neuron expression of mutant hKCNT1 channels significantly reduce average crawling distance compare to controls. (**c**) Representative images of pupal morphology. Larvae expressing mutant hKCNT1 channels show bent pupa shape. At least twenty larvae were used per genotype (10

387 males and 10 females). All larvae were reared at 22 °C for 5 days then at 25 C° for 2

388 days. Error bars show S.E.M. **P < 0.01 one-way ANOVA, Turkey HSD *post hoc test*.

389



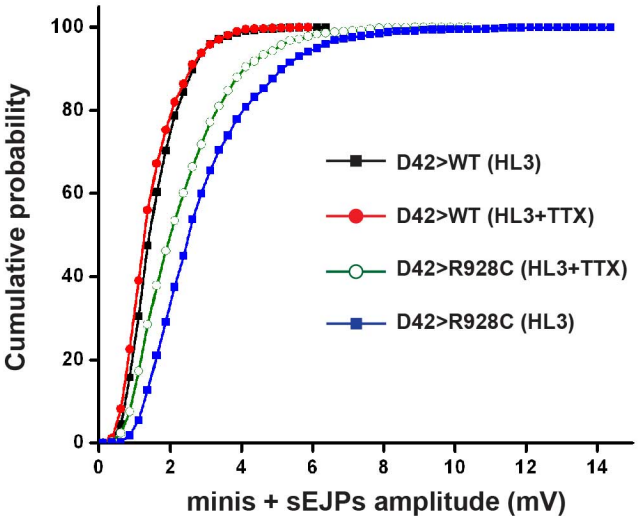
390

391 **Supplementary Figure 4: Motoneuronal expression of mutant hKCNT1 channels**

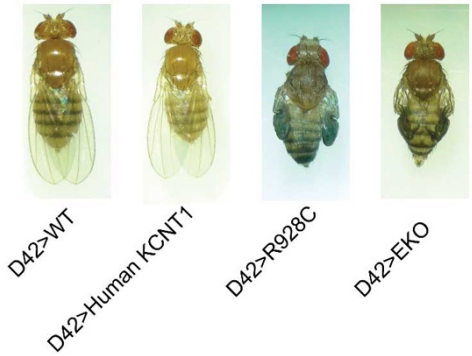
392 **did not affect membrane resting potential.** Both controls and larvae expressing

393 mutant hKCNT1 channels in motoneurons have similar membrane resting potentials.

394



Supplementary Figure 5: Blocking voltage-gated Na^+ channels with TTX reduced the number of extremely large sEJPS but did not abolish sEJPs in larvae expressing mutant hKCNT1 channels. Cumulative probability plot of minis plus sEJPs shows that TTX reduced the number of the extremely large sEJPs (>10 mV), but did not eliminate the sEJPs.



Supplementary Figure 6: neuronal expression of the mutant KCNT1 Channels causes folded wing phenotype. Representative images of adult fly wing morphology. All larvae were reared at 18 °C until eclosion.

407 **Supplementary Movie legends**

408 **Movie 1: Muscle expression of mutant hKCNT1 channels hinders larvae crawling**
409 **activity.** Representative crawling activity assay videos of controls and mutant KCNT1
410 larvae. Larvae expressing mutant hKCNT1 channels in muscle show significant
411 reduction in crawling activity compared to controls. Twenty larvae (ten males and ten
412 females) were used per genotype, and all larvae were aged at 25 °C prior to testing.

413

414 **Movie 2: GABAergic expression of mutant hKCNT1 channels increase the number**
415 **of seizing flies and seizing duration in adult flies.** Representative bang-sensitivity
416 behavioral assay videos. Flies expressing mutant hKCNT1 channels in inhibitory
417 neurons exhibit seizure phenotypes, such as spinning in the bottom of the vial or flip on
418 their back and remain at that state for one second or more. At least 40 flies were used
419 per genotype, and all larvae were aged at 18 °C until they enclosed then adult flies are
420 aged between 7-10 days at 25 °C prior to testing.

421

422 **Movie 3: Motoneuronal expression of mutant hKCNT1 channels increase the**
423 **number of seizing flies and seizing duration in adult flies.** Representative bang-
424 sensitivity behavioral assay videos. Flies expressing mutant hKCNT1 channels in
425 motoneurons exhibit seizure phenotypes, such as spinning in the bottom of the vial or
426 flip on their back and remain at that state for one second or more. 120 flies were used
427 per genotype, and all larvae were aged at 18 °C until they enclosed then adult flies are
428 aged for two weeks at 25 °C prior to testing.

429

Movie 4: Larvae expressing mutant hKCNT1 channels in motoneurons shows uncoordinated contraction of left and right abdominal muscle hemisegments. Representative crawling activity assay videos of controls and mutant KCNT1 larvae. Larvae expressing mutant hKCNT1 channels in motoneurons show significant reduction in crawling activity compared to controls, due to unsynchronized muscle contractions. Twenty larvae (ten males and ten females) were used per genotype, and all larvae were aged at 22 °C for 5 days and at 25 °C for two days prior to testing.

Movie 5: Ca^{2+} waves in larvae expressing mutant hKCNT1 channels in motoneurons shows uncoordinated VNC abdominal ganglia firing. Representative Ca2+ waves assay videos of controls and mutant hKCNT1 larvae. Larvae expressing mutant KCNT1 channels in motoneurons show significant increase in unsynchronized abdominal ganglia firing. Ca^{2+} waves recording was at 2 frames per second and movie is showing 4X the original speed. Four larvae were used per genotype, and all larvae were aged at 22 °C for 5 days and at 25 °C for two days prior to testing.

447 **Methods**

448 **Fly Strains.**

449 Fly cultures kept at 12-h light/dark cycle on standard cornmeal food. The following
450 *Drosophila* strains were used: *D42-GAL4*, *OK6-GAL4*, *GAD1-GAL4*, *GAL80^{ts}*, *Mhc-*
451 *GAL4*, *8622*, and *UAS-GCaMP6S* were obtained from Bloomington Stock Center. *UAS-*
452 *Kir2.1* was gift from G. Davis, *UAS-EKOX2*, gift from B. White, and UAS-KCNT1
453 transgenic lines, gift from L. Dibbens.

454

455 **Larvae crawling behavior assay and analysis.**

456 Wandering third-instar larvae were washed with double distilled water and placed on
457 150mm petri dish containing 1% agarose. The larvae locomotion behavior was recorded
458 for 1 min at 30 frames/s by Fujinon DF6HA-1B camera and FlyCapture software. Videos
459 were analyzed using ImagJ and wrMTrck plugin as previously descried[59].

460

461 **Calcium imaging.**

462 Third-instar larvae were dissected and ventral nerve cord, which contains motoneurons,
463 was viewed using Olympus BX61 compound microscope with 10X air lens. Calcium
464 waves were recorded using ORCA-R^2 CCD camera (Hamamatsu) and CellSens
465 Dimension 1.9 software. ImageJ software was used for calcium waves analysis.

466

467 **Electrophysiology**

468 Recordings were made from muscle 6 in abdominal segments 3 and 4 from third-instar
469 larvae as previously discribed[35,36]. Recordings were made with a modified HL3 saline[60]

470 containing 70mM NaCl, 5mM KCl, 10mM $MgCl_2$, 10mM $NaHCO_3$, 5mM trehalose,
471 115mM sucrose, 5mM HEPES, and 1.2mM $CaCl_2$. For true mini was recorded with HL3
472 solution plus 1 μM TTX and 10 μM $CdCl_2$. Recording microelectrodes were fabricated
473 using P2000g puller (Sutter instruments) and filled with 3M KCl. Signals were recorded
474 by Axoclamp B2 amplifier and pCLAMP 6 software (Molecular devices). Severed
475 segmental nerve was sucked by stimulation microelectrode and stimulated by Master-8
476 pulse generator and iso-flex stimulus isolator (A.M.P.I.).
477
478 **Fly bang-sensitive assay**
479 Experiment was done as previously descried with some modification[29,30]. Briefly, flies
480 were collected immediately after eclosion, aged for 13 days at 25 °C, and mechanically
481 stimulated with mini vortexer (VWR Scientific Products) at power setting 10. Flies
482 behavior was recorded by SONY (HDR-SR11) camcorder. Flies that flipped on their
483 back and remained at that position for at least one second were considered to be
484 exhibiting seizure activity.
485
486 **Immunocytochemistry.**
487 Wandering third-instar larvae were dissected in HL3 solution and fixed for 2h in
488 4%paraformaldehyde. Fixed larvae were washed 3X 15 min each with PBST (0.2
489 TritonX-100). Larvae were incubated overnight at 4 °C with primary antibody with the
490 appropriate dilution. The following antibodies were used: guinea pig anti-Shaker 1:500
491 (gift from Kyunghee Koh), rabbit HRP-488 1:500. Alexa-conjugated secondary

492 antibodies (1:500) were used. Images were acquired with Leica SP2 confocal

493 microscope using a 63X oil immersion objective.

494

495 **Statistical analysis.**

496 Data were analyzed as described in the figure legends using Origin software version

497 6.0.

498

499

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656
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