Title: Contribution of EAG to excitability and potassium currents in *Drosophila* larval motoneurons

Authors and author addresses: Subhashini Srinivasan\(^1,2\), Kimberley Lance\(^1,2\), Richard B. Levine \(^1,2,3\)
\(^1\)Department of Neuroscience, \(^2\)Graduate Program in Physiological Sciences, \(^3\)Department of Physiology, University of Arizona, Tucson, AZ 85721, USA

Running head: Role of EAG channels in motoneuron excitability

Corresponding author with complete address:
Richard B. Levine
Email address: rbl4@email.arizona.edu
Department of Neuroscience
University of Arizona
PO Box 210077
Tucson AZ 85721-0077
520.621.6655 (phone)
520.621.8282 (fax)
Abstract

Diversity in the expression of potassium channels among neurons allows a wide range of excitability, growth and functional regulation. Ether-a-go-go (EAG), a voltage-gated K\(^+\) channel, was first characterized in *Drosophila* mutants by spontaneous firing in nerve terminals and enhanced neurotransmitter release. Although diverse functions have been ascribed to this protein, its role within neurons remains poorly understood. The aim of this study was to characterize the function of EAG *in situ* in *Drosophila* larval motoneurons. Whole-cell patch-clamp recordings performed from the somata revealed a decrease in I\(_{A_v}\) and I\(_{K_v}\) K\(^+\) currents in *eag* mutants and with targeted eag RNAi expression. Spontaneous spike-like events were observed in *eag* mutants but absent in wild type motoneurons. Thus, our results provide evidence that EAG represents a unique K\(^+\) channel contributing to multiple K\(^+\) currents in motoneurons helping to regulate excitability, consistent with previous observations in the *Drosophila* larval muscle.

Key words: Spontaneous firing, Potassium channel subunit, Heteromultimer
Introduction

The wide variety, density, and distribution of $K^+$ channels in neurons help to regulate the resting membrane potential, action potential shape, firing rate, and synaptic release from nerve terminals. In recent years, a wealth of information has emerged regarding the novel interactions among ion channels and their ability to influence signaling pathways (for reviews: Kaczmarek 2006). Ether-a-go-go (EAG), a member of the voltage-gated potassium channel family, was identified as a *Drosophila* mutant exhibiting abnormal leg shaking phenotype and later shown to display repetitive spontaneous firing of nerve terminals (Kaplan and Trout 1969; Ganetzky and Wu 1983).

DNA sequence and protein alignment analysis of EAG reveal a putative $K^+$ channel subunit, which is similar to the Shaker family of $K^+$ channels. Both form tetramers and each monomer has six putative transmembrane domains, including a voltage-sensing domain (S4) and a pore-forming domain that is selective to $K^+$. *In vivo*, *Drosophila shaker* and *eag* double mutants display synergistic effects in increasing spontaneous activity at the larval neuromuscular junction. Earlier studies using voltage-clamp recordings from *Drosophila* larval muscles demonstrated a reduction of $I_{A_N}$ and delayed rectifier $I_{K_r}$ currents in *shaker* and *eag* mutants respectively (Wu et al. 1983). Different allelic mutants of *eag* were later shown to reduce different components of $K^+$ currents (Zhong and Wu 1991 and 1993). *Ex vivo* voltage-clamp recordings from *Xenopus* oocytes containing *eag* cDNA revealed outward sustained $K^+$ current (Robertson et al. 1996). Coexpression of Shaker and EAG RNA in oocytes led to faster inactivation of transient currents, suggesting a possible heteromultimeric channel assembly. However, this association is dependent on the concentration of the RNA used and the developmental
time of the oocytes (Chen et al. 1996, 2000 and Tang et al. 1998). Both these approaches have led to the speculation that EAG could form heteromultimers with other $K^+$ channels. A definitive proof of heteromultimeric EAG channel formation in vivo remains to be explored.

Unlike the Shaker family, EAG has a cyclic-nucleotide binding domain in the carboxy (C) terminus similar to cyclic-nucleotide gated channels (Warmke et al. 1991; Guy et al. 1991). In addition to functioning as a voltage-gated ion channel, EAG displays numerous non-ion conducting roles. Modulation of multiple potassium currents by cGMP and calmodulin antagonist (W7) is reduced in EAG mutants (Zhong and Wu 1993). The C terminus of EAG contains a calcium/calmodulin dependent protein kinase II binding site. Similar to $eag$ mutants, CAMKII inhibitor (ala) also increases spontaneous repetitive firing (Griffith et al. 1994). $\text{Ca}^{++}$ or calmodulin is necessary for binding of CaMKII to EAG. Phosphorylation of EAG by CaMKII regulates EAG function (Wang et al. 2002). In turn, the binding domain of CaMKII in EAG dislodges the autoinhibition of CaMKII, thereby enabling it to be stably bound to EAG for a long time even in the absence of substrates (Sun et al. 2004). In addition to the above, the voltage sensor of non-conducting EAG channels has been shown to activate p38 mitogen-activated protein kinase (MAPK) signaling leading to cell proliferation (Hegle et al. 2006).

Yet another novel function of EAG is its emerging role in transcription. $eag$ transcripts are alternatively spliced to produce an 80kDa protein (EAG80), which contains N and C termini leaving the channel forming transmembrane regions of the protein. Synthesis of EAG80 can be triggered in vitro by calcium influx, and by
activation of PKA or PKC through MAPK signaling bringing about changes in cell morphology. The C terminus of EAG has a nuclear localization signal that enables it to translocate to the nucleus. This nuclear translocation although necessary for MAPK signaling, does not serve directly as a transcriptional activator (Sun et al. 2009). Higher levels of EAG are found in cancerous cells linking EAG to cell cycle regulation and proliferation (for reviews: Camacho 2006).

EAG represents a class of ion channels whose functional role in cellular excitability and regulation remains less understood. A wealth of information regarding the varied potential functions of EAG has been obtained from experiments using oocytes and cell culture systems. It is yet to be determined if these functional roles are observed in the nervous system. Therefore, the aim of this study is to provide an electrophysiological characterization of the role of EAG in an identified Drosophila larval motoneuron using in situ whole-cell patch-clamp technique and genetic manipulations. Our results indicate that EAG contributes to diverse K+ currents, including transient and sustained voltage-activated currents and in its absence increases excitability.
Methods

Drosophila stocks

*even-skipped* promoter GAL4 lines, w*;*noc/sco;RRA-GAL4, UAS-CD8GFP

(Fujioka et al. 2003) and a flip-out strategy using w*;*RN2-GAL4 (w*; RN2-GAL4, UAS-
mCD8-GFP; ACT5C<<CD2<<GAL4,UAS-FLP, Hartwig et al. 2008) were used to label

aCC and RP2 motoneurons in the larval ganglion. w*,ELAV-GAL4;+;UAS-GFP was

used to misexpress UAS-eag RNAi in all neurons. UAS-eag RNAi (Transformant ID: 9127) and UAS-Dicer 1 (Transformant ID: 24667) were obtained from the Vienna

Drosophila RNAi Center. For misexpression studies, UAS-eag RNAi and UAS- Dicer 1

stocks were combined and then crossed to appropriate GAL4 lines. w*; P{tubP-

GAL80{18}20; TM2/TM6B, Tb^1 (7019, Bloomington Stock Center, Indiana) was used

block GAL4 expression. *eag* mutants, Df (1)eag *x-6/FM7a, lethal; Dp(1:2)X-6/SM6b,Cy

(hereafter, *eag* *x-6*) and In(1)sc29, *wa* (hereafter, *eag* *sc29*) were obtained from Dr. B.

Ganetzky (University of Wisconsin, Madison). Canton-S and w*1118* were used as wild

type control for mutants and appropriate parental GAL4 lines were used as controls for

RNAi studies.

Electrophysiology

Wandering third instar *Drosophila* larvae were dissected in Ca++-A solution (containing,
in mM, 118 NaCl, 2 NaOH, 2 KCl, 4 MgCl2, 5 trehalose, and 5 HEPES, pH 7.1-7.2 and

osmolarity adjusted to 295mOSM using sucrose) and used as the external recording

solution during electrophysiological experiments. Procedures for dissection and

preparations for performing electrophysiological experiments were carried out as
described previously (Srinivasan et al. 2012). Male larvae from eag mutants were used for electrophysiological experiments. aCC motoneurons in the first thoracic segment were identified by intracellular dye filling using Dextran, Rhodamine 3000 MW (Catalog: D-3307, life technologies). The position of the cell body in the ganglion and the bilateral dendritic arborization and ipsilateral axon were used to confirm aCC (Srinivasan et al. 2012).

**Protocols and pharmacology**

Glass micropipettes of 2.5-5MΩ (in bath) were filled with potassium gluconate internal solution (containing, in mM, 120 K gluconate, 20 KCl, 1 MgCl₂, 0.5 CaCl₂, 5 EGTA, and 10 HEPES; pH adjusted to 7.1-7.2 and osmolarity to 285-290 mOsm using glucose). Whole-cell path-clamp recordings were performed using the Multiclamp 700B amplifier (Axon Instruments, Molecular Devices, Sunnyvale, CA). Clampex acquisition software (PClamp 10, Molecular Devices) was used to initiate and record voltage and current commands. Voltage-clamp protocols were used to isolate voltage-dependent K⁺ currents. The cells were held at -80mV and command steps of 20mV were provided from -120mV up to 60mV in 200ms duration. To visualize sustained K⁺ currents in isolation, (Fig. 2) the cells were held at -20mV to inactivate the transient currents. Measurements of sustained currents (graphs in Fig. 2, 3 & 4) were made near the end of the 200ms long voltage step from cells held at -80mV, so that the voltage dependence of activation could be observed more accurately. A current-clamp protocol was used to study firing properties. A series of 10pA, 500ms current steps ranging from -10pA to 100pA were administered. To measure firing frequency, since resting membrane potential varied
across cells, a bias current was injected to normalize the resting membrane potential to -60mV in all cells. Extended periods of motor neuron activity were monitored using the gap-free protocol and bias current was used to hold the cell at different membrane potentials. Na⁺ currents were blocked using external application of 1µM Tetrodotoxin (Sigma-Aldrich, St. Louis, MO). Ca²⁺ currents were blocked using external application of 500µM cadmium chloride (Acros Organics, Belgium) in TTX containing Ca²⁺- A solution. Chemical reagents were obtained from Sigma-Aldrich, unless specified.

**Quantification**

Input resistance was measured using the most hyperpolarizing voltage step (-40mV) in voltage-clamp experiments using Clampfit 10.1 (Molecular Devices). Values of input resistance were used to perform leak subtraction. All voltage-clamp records were leak subtracted unless otherwise indicated in the text. Series resistance was measured using the charging transient generated with a hyperpolarizing voltage step. Current densities are reported.

**Confocal microscopy**

For imaging isolated RP2 motoneurons, w⁻; RN2-GAL4, UAS-mCD8-GFP;

ACT5C<<CD2<<GAL4,UAS-FLP was crossed with w¹¹¹⁸ to obtain w⁻; RN2-GAL4, UAS-mCD8-GFP/+; ACT5C<<CD2<<GAL4,UAS-FLP/+. To visualize the effect of EAG RNAi manipulations on motoneuron dendrites, w⁻; RN2-GAL4, UAS-mCD8-GFP;

ACT5C<<CD2<<GAL4,UAS-FLP was crossed with w⁻;UAS-EAG RNAi; UAS- Dicer 1 to obtain w⁻; RN2-GAL4, UAS-mCD8-GFP/ UAS-eagRNAi;
ACT5C<<CD2<<GAL4,UAS-FLP/UAS-Dicer1. Third instar larvae were dissected in phosphate buffer saline (PBS). The preparation was mounted in 80% glycerol (Sigma-Aldrich, diluted in dH2O). Live images were taken with a Ziess 510 Meta Laser Scanning Confocal Microscope at 40x with a 1.8 zoom. Images post processed using Ziess LSM Image Browser Version 4.2.0.121 (Fig. 3 G & H).

Temperature-sensitive experiments

w−; P{tubP-GAL80\textsuperscript{ts}}\textsuperscript{20};RRA-GAL4,UAS-CD8GFP females were crossed to w−;UAS-eag RNAi;UAS-Dicer 1 males. For L3 knockdown experiments, progeny were raised at 18ºC until the early-mid third instar and then transferred to 30ºC. Recordings were performed 1.5 days later in wandering late third instar. In the chronic experiments, eag RNAi was expressed throughout development by raising the progeny at 30ºC until the wandering third instar stage. Parental line (w−; P{tubP-GAL80\textsuperscript{ts}}\textsuperscript{20};RRA-GAL4, UAS-CD8GFP) reared at the same temperatures served as the control. GFP expression in the late third instar larvae was used as an indicator for GAL80 suppression (Fig. 4A & B).

Statistical analysis

Student t-test was used to recognize significance (Excel 4.0, Microsoft, Redmond, WA). Two-way ANOVA followed by Bonferroni post-test was used to compare multiple genotypes and command potential (GraphPad Prism 5). Current densities and standard error of mean are reported.
Results

EAG contributes to transient $K^+$ currents

Our overall aim was to characterize the \textit{in vivo} role of EAG in the nervous system by performing \textit{in situ} whole-cell patch-clamp recordings on the somata of aCC motoneurons in the first thoracic segment (T1aCC). Active and passive properties of this motoneuron have been described previously (Srinivasan et al. 2012). We tested the contribution of EAG to $K^+$ currents using two well studied mutants in the larval neuromuscular junction, \textit{eag} \textsuperscript{x-6} and \textit{eag} \textsuperscript{sc29}. \textit{eag} \textsuperscript{x-6} is a $\gamma$-ray induced mutation resulting in the deletion of the chromosomal fragment between the locus 13A1-2 and 13E4-8 in the X chromosome and insertion of the fragment at locus 21E in the second chromosome resulting in two DNA bands in \textit{in situ} hybridization (Drysdale et al. 1991). \textit{eag} \textsuperscript{sc29} is an inversion mutation resulting in a break point at 12F-13A in the X chromosome (Drysdale et al. 1991). Northern blot analysis from wild type, \textit{eag} \textsuperscript{x-6} and \textit{eag} \textsuperscript{sc29} revealed a10kb fragment present in wild type extracts was missing in the two mutants, but smaller fragments were observed (Drysdale et al. 1991). EAG protein is observed in the synaptic neuropil in wild type larvae, but absent in \textit{eag} \textsuperscript{sc29} (Sun et al. 2004).

In the \textit{Drosophila} larval muscle, allele specific reduction of \textit{eag} resulted in a decrease in $I_{A,V}$, $I_{A(Ca)}$, $I_{K,v}$ and $I_{K(Ca)}$, with \textit{eag} \textsuperscript{x-6} selectively reducing $I_{A,V}$ and $I_{K,v}$ (Zhong and Wu 1991). Whole-cell patch-clamp recordings performed on the somata of aCC motoneurons revealed total transient $K^+$ currents clearly visible in wild type, Canton-S (CS), \textit{eag} \textsuperscript{x-6} and \textit{eag} \textsuperscript{sc29} when depolarized above -20mV (Fig. 1A & C). Two-way ANOVA comparing total transient $K^+$ current in CS, \textit{eag} \textsuperscript{x-6} and \textit{eag} \textsuperscript{sc29} revealed overall
main effects of genotype \([F(2, 130) = 99.03, p< 0.0001]\) and command potential \([F(9, 130) = 378.9, p< 0.0001]\) (Fig. 1A & C). The Bonferroni post-test revealed that \(eag^{sc29}\) showed a significant reduction \((p<0.001)\) from 0 to 60mV compared to CS (Fig. 1C). No significant reduction was observed between CS and \(eag^{x-6}\) (Fig. 1A & C). However, a significant difference \((p<0.001)\) was observed from 0 to 60mV between \(eag^{sc29}\) and \(eag^{x-6}\).

We then tested the specific contribution of EAG to voltage-activated transient K\(^+\) current \((I_{Av})\). \(I_{Av}\) was isolated by bath application of cadmium chloride to block calcium channels in the same cell in all genotypes (Fig. 1B). \(I_{Av}\) currents were visible in response to command steps from -20 through 60mV (Fig. 1B). Similar to the total transient K\(^+\) currents, two-way ANOVA comparing \(I_{Av}\) in CS, \(eag^{x-6}\) and \(eag^{sc29}\) revealed overall main effects of genotype \([F(2, 130) = 65.8, p< 0.0001]\) and command potential \([F(9, 130) = 205.5, p< 0.0001]\) (Fig. 1B & D). The Bonferroni post-test revealed that \(eag^{sc29}\) showed a significant reduction \((p<0.001)\) from 0 to 60mV and \(eag^{x-6}\) also showed a reduction at 40 and 60mV \((p<0.001)\) compared to CS (Fig. 1D). In addition, a significant difference \((p<0.05)\) was observed from 0 to 60mV between \(eag^{sc29}\) and \(eag^{x-6}\).

We estimated the amplitude of calcium-activated transient K\(^+\) currents by subtracting \(I_{Av}\) currents from the total K\(^+\) currents. There were overall effects of both genotype \([F(2, 130) = 17.40, p< 0.0001]\) and command potential \([F(9, 130) = 78.71, p< 0.0001]\). The Bonferroni post-test indicated that there were significant differences \((p<0.05)\) between \(eag^{sc29}\) and control from 20 to 60mV, but no significant differences were observed between \(eag^{x-6}\) and control. Similarly, there were significant differences
between \( {eag^{sc29}} \) and \( {eag^{x-6}} \) from 20 to 60mV (\( p<0.001 \)) (Fig. 1E). Thus, \( I_{A(Ca)} \) was significantly reduced in \( {eag^{sc29}} \) mutants. Total transient \( K^+ \) current and \( I_{A(Ca)} \) in \( {eag^{x-6}} \) appeared to plateau at 60mV (Fig. 1C & E). It is possible that the reversal potential of calcium currents is altered in this mutant.

**EAG contributes to sustained \( K^+ \) currents**

Total sustained \( K^+ \) current was isolated from the same cell using a -20mV voltage-clamp protocol to inactivate transient \( K^+ \) currents (Fig. 2A). Sustained \( K^+ \) currents were activated from 0 to 60mV (see methods). Two-way ANOVA comparing total sustained \( K^+ \) current in CS, \( {eag^{x-6}} \) and \( {eag^{sc29}} \) revealed overall main effects of genotype \( [F(2, 130) = 40.16, p< 0.0001] \) and command potential \( [F(9, 130) = 253.7, p< 0.0001] \) (Fig. 2A & C). The Bonferroni post-test revealed that total sustained \( K^+ \) current was significantly reduced (\( p<0.001 \)) from 20 to 60mV in \( {eag^{sc29}} \) compared to CS (Fig. 2A & C). A significant reduction (\( p<0.001 \)) in total sustained \( K^+ \) current was observed in \( {eag^{sc29}} \) compared to \( {eag^{x-6}} \) from 20 to 60mV (Fig. 2C).

Further isolation of voltage-dependent delayed rectifier current (\( I_{Kv} \), after application of cadmium chloride) revealed overall effects of genotype \( [F(2, 130) = 27.51, p<0.0001 ] \) and command potential \( [F(9, 130) = 94.53, p< 0.0001] \) (Fig. 2B). The Bonferroni post-test confirmed that \( I_{Kv} \) was significantly reduced in \( {eag^{sc29}} \) compared to CS from 20 to 60mV (\( p<0.001 \)) (Fig. 2D). \( {eag^{x-6}} \) showed no clear reduction in the delayed rectifier \( K^+ \) current. A significant difference (\( p<0.001 \)) was also observed from 20 to 60mV between \( {eag^{sc29}} \) and \( {eag^{x-6}} \). The level of the calcium sensitive component of
IK was estimated through subtraction as described above. Again, there were overall
effects of both genotype $[F(2, 130) = 6.944, p< 0.01]$ and command potential $[F(9, 130) = 65.90, p< 0.0001]$. The Bonferroni post-test revealed that there was a significant
reduction in $eag^{sc29}$ as compared to CS, only at 40mV ($p<0.05$) (Fig. 2E). There was no
significant differences observed between CS and $eag^{x-6}$. Taken together, $eag^{sc29}$
demonstrated a reduction in total transient and sustained K$^+$ currents and also in $I_{A_V}$, $I_{A(Ca)}$
and $I_{K_V}$. The $eag^{x-6}$ allele, on the other hand, showed a reduction only in $I_{A_V}$.

**Targeted reduction of EAG in motoneurons**

To further study the effect of EAG on potassium currents only in motoneurons,
eag RNAi was misexpressed in even-skipped promotor expressing GAL4 line, RRA. No
reduction in total transient K$^+$ current was observed when eag RNAi was misexpressed
compared to the GAL4 control (Fig. 3A). However, further isolation of $I_{A_V}$ current
revealed that eag RNAi caused a reduction in the current from -20 up to 60mV (Fig. 3C).
Interestingly, $I_{A(Ca)}$ current was significantly increased in eag RNAi misexpression
compared to control (Fig. 3E). In effect, eag RNAi misexpression caused reduction
similar to $eag^{x-6}$ in transient K$^+$ current. eag RNAi did not cause a significant reduction
in total sustained K$^+$ current (Fig. 3B), however, a significant reduction was observed in
$I_{K_V}$ from 0 to 60 mV (Fig. 3D). No reduction in $I_{K(Ca)}$ was observed (Fig. 3F). We did not
observe any difference in the gross morphology of dendrites and cell body of
motoneurons with these manipulations, although dendritic branching has yet to be
analyzed in depth (Fig. 3G & H).
Embryonic contribution of EAG to $K^+$ currents

Reduction in EAG function in mutants and eag RNAi manipulations lead to increased excitability and perhaps other changes throughout development. Therefore, functional changes that are measured late in the larval stage may be influenced by the embryonic role of EAG protein. To eliminate the embryonic role, eag RNAi was expressed under the control of RRA-GAL4 and a temperature sensitive GAL80, a blocker of GAL4, was used to conditionally block expression of eag RNAi in embryonic and early larval stages. Temperature-sensitive GAL80 functions at lower temperatures ($18^\circ C$) to prevent GAL4 expression, but is blocked at higher temperatures ($30^\circ C$), thereby allowing GAL4 expression (McGuire et al. 2003). Thus, by controlling the rearing temperature, eag RNAi expression was allowed only in the third instar larval stage and not in the embryonic, first and second instar larvae. GFP expression was absent at $18^\circ C$, confirming the activation of GAL80, and present at $30^\circ C$, confirming suppression of GAL80 (Fig. 4A & B).

$K^+$ currents were measured from larvae in which eag RNAi was expressed chronically (from embryo to L3) and only in the late larval stage (shifted at mid L3) and compared to controls (raised in a similar temperature shift paradigm as the larval knock down). Two-way ANOVA comparing total transient $K^+$ current in chronic and larval eag RNAi knockdown with control revealed overall main effects of genotype [$F(2, 220) = 6.189, p< 0.01$] and command potential [$F(9, 220) = 490.7, p< 0.0001$] (Fig. 4C). The Bonferroni post-test revealed that total transient $K^+$ current was significantly reduced ($p<0.05$) in larval eag RNAi compared to control from 40 to 60mV. As in the first set of eag RNAi experiments, however, chronic eag RNAi showed no significant reduction in
transient $K^+$ current compared to control. A significant reduction ($p<0.05$) was also observed between chronic and larval eag RNAi knockdown at 60mV (Fig. 4C).

Looking specifically at the voltage-dependent component of the transient current, overall effects of genotype $[F(2, 220) = 11.68, p<0.0001]$ and command potential $[F(9, 220) = 412.5, p<0.0001]$ on $I_{A_{V}}$ were observed between chronic or larval eag knock down compared to control. The Bonferroni post-test indicated that $I_{A_{V}}$ was significantly reduced ($p<0.01$) in chronic knock down at 60mV and larval knock down at 40 and 60mV compared to control. No difference in $I_{A_{V}}$ was observed between chronic and larval eag RNAi knockdown (Fig. 4E). In addition, no reduction was observed in $I_{A(Ca)}$ in these temperature manipulations (data not shown).

Overall effects of genotype $[F(2, 220) = 9.102, p<0.001]$ and command potential $[F(9, 220) = 455.4, p<0.0001]$ on total sustained $K^+$ current were observed between chronic and larval eag knock down when compared to control. The Bonferroni post-test indicated that total sustained $K^+$ current was significantly reduced ($p<0.001$) in both chronic and larval knock down at 40 and 60 mV (Fig. 4D). No difference was observed between chronic and larval eag knock down. $I_{K_{v}}$ also showed overall effects of genotype $[F(2, 220) = 7.215, p<0.0001]$ and command potential $[F(9, 220) = 267.3, p<0.0001]$ in these temporal manipulations. The Bonferroni post-test indicated a significant reduction ($p<0.001$) at 60mV between chronic and larval eag RNAi manipulations. No reduction was observed in chronic and larval eag RNAi manipulations compared to control (Fig. 4F). Both chronic and larval manipulations reduced $I_{K(Ca)}$ significantly only at the 40mV command (data not shown).
Taken together, these results demonstrate the role of EAG in larval stages without its embryonic contribution. Failure to observe a reduction in $I_{Kv}$ in the larval manipulation suggests a possible requirement of EAG during development. Surprisingly, total transient $K^+$ current was reduced in the larval knockdown but not in the chronic knockdown, suggesting embryonic compensation. $I_{Av}$ was reduced in all manipulations, demonstrating that EAG contributes acutely to $K^+$ currents in larval T1aCC. We cannot rule out the possibility that there was some degree of $K^+$ current compensation by other $K^+$ channels in larval eag RNAi knockdown, however, so our measure of $K^+$ current reduction in larval eag RNAi manipulations may be an underestimation.

**EAG influences firing properties in motoneurons**

We observed a decrease in diverse $K^+$ currents in eag mutants and in eag RNAi manipulation. We hypothesized the reduction in multiple $K^+$ currents may influence firing behavior of motoneurons. A current-clamp protocol was used to obtain firing frequency plots from control lines and eag mutants. Depolarizing steps of current injection were administered to CS (control), to evoke action potentials. The voltage threshold for firing was $-34.85 \pm 2.5$ mV. The average resting membrane potential of T1aCC was $-49.2 \pm 1$ mV, the average input resistance was $383.16 \pm 49 \Omega$ (Table 1). A delay to the first spike was observed following current injection (Fig. 5A). Baseline synaptic activity was also observed (Fig. 5A). The average resting membrane potential in eag $x^{-6}$ and eag $x^{29}$ was more depolarized than in wild-type at $-34.5$ mV and $-37.7$ mV.
respectively, but the average input resistance was not affected (396.73 ± 88MΩ and
378.79 ± 10MΩ; Table 1).

In the most striking phenotype of eag<sup>x-6</sup> and eag<sup>x-29</sup>, large spontaneous spike-like
events were observed, intermittently with little or no current injection, in some recordings
(Table 1; dotted black box in Fig. 5B & C). These spontaneous events did not cause an
increase in the frequency of evoked firing following current injection (Fig. 5G, current
step 70pA). However, a decrease in the delay to first spike following current injection
was observed when spontaneous spike-like events occurred in mutants (Fig. 5B & C).
The spontaneous events in eag mutants were sometimes broader in shape, similar to those
observed in Fig. 5E. Hyperpolarizing current injection increased the amplitude of the
spontaneous events, as expected for excitatory postsynaptic potentials or action
potentials. In addition, the frequency of the events decreased with hyperpolarization,
suggesting a contribution of voltage-gated currents (Fig. 6B). In the example from an
eag mutant shown in figure 6B, action potentials of ~10mV in amplitude with a
prominent after-spike hyperpolarization were observed at a depolarized membrane
potential of -20mV. Upon hyperpolarizing the cell to -60mV, spontaneous events of ~
11mV in amplitude were observed with no visible after-spike hyperpolarization. Such
spontaneous events were never observed in wild type, CS, at -60mV. At - 95mV in the
mutant cell, a further increase in amplitude to about 18mV and a decrease in frequency
were observed (Fig. 6B). In some recordings from the mutants, large EPSPs (Table 1)
and an increased frequency of EPSPs were also observed (Fig. 6A). As expected of
EPSPs, these events increased in amplitude, but did not decrease in frequency with membrane hyperpolarization.

The spontaneous events in mutants could reflect increased synaptic drive and/or altered intrinsic excitability of the motoneuron. We first attempted to phenocopy the eag mutants by using ELAV-GAL4 to express eag RNAi in all neurons. Similar to eag mutants, ELAV::eag RNAi displayed a more depolarized resting membrane potential of -37± 3mV compared to control, but the average input resistance was not affected (286.93± 8MΩ; Table 1). Spontaneous events, similar to eag mutants, were also observed in ELAV::eag RNAi manipulations (Table 1; dotted black box in Fig. 5E). ELAV::eag RNAi motoneurons displayed a non-significant increase in frequency of evoked firing compared to control (Fig. 5F).

To help distinguish the effects of synaptic input from intrinsic changes in excitability, eag RNAi was driven in a small population of motoneurons by RRA-GAL4. CS and RRA-GAL4 controls displayed identical firing frequency characteristics (Fig. 5G & 5H). No significant change in resting membrane potential or input resistance was observed between RRA-GAL4 control and RRA::eag RNAi. Spontaneous spike-like events were not observed in RRA::eag RNAi motoneurons and evoked firing frequency was comparable to control. However, in both ELAV and RRA::eag RNAi manipulations, large EPSPs were sometimes observed (Table 1, black box in Fig. 5F). Table 1 represents the variability in the frequency of physiological phenotypes.
EAG mutants display increased frequency and amplitude of EPSPs during rhythmic activity.

In some recordings it was possible to observe extended periods of rhythmic synaptic input to motoneurons (Fig. 6A). In CS, bouts of rhythmic synaptic activity lasting 3-4s with compounding EPSPs of about 6mV in amplitude (black box), and baseline synaptic activity composed of small EPSPs roughly 2mV in amplitude (dotted black box), were observed. The latter were also observed in the absence of rhythmic activity (as shown in Fig. 5A). In eag^{x-6}, longer bouts of rhythmic activity (6-8s) with larger EPSPs (about 12mV, black box) and a higher frequency of baseline synaptic input were observed (dotted black box).
Discussion

EAG contributes to diverse K+ currents

eag mutants, eag \(^{sc29}\) and eag \(^{x-6}\), and eag RNAi manipulations showed a reduction in I\(_{Av}\) and I\(_{Kv}\). An increased excitability was also observed in eag mutants. K+ currents play important roles in regulating excitability. I\(_{Av}\) currents are important in maintaining the shape and timing of action potentials. The delay to first spike observed in wild type T1aCC was reduced in eag mutants displaying spontaneous spike-like events. I\(_{Av}\) current is responsible for the delay to spike observed in larval motoneurons (Choi et al. 2004).

Shal, a voltage-activated K+ channel subunit, contributes to I\(_{Av}\) currents in the adult flight motoneuron, MN5, and is probably the predominant I\(_{Av}\) current in the somatodendritic compartment of embryonic and thoracic larval aCC and RP2 motoneurons (Ryglewski and Duch 2009; Baines and Bate 1998; Schaefer et al. 2010). A prominent reduction in I\(_{Av}\) and a decreased delay to spike in Shal RNAi experiments has been observed (Schaefer et al. 2010; Srinivasan et al. 2012). In addition to Shal, EAG subunit also contributes to the I\(_{Av}\) current (present results and Zhong and Wu 1991). However, unlike Shal, eag manipulations also reduced I\(_{Kv}\) (present results and Zhong and Wu 1991).

Shaker, in addition to Shal, contributes to I\(_{Av}\) in the adult motoneuron, MN5 (Ryglewski and Duch 2009), but ordinarily not in the somatodendritic compartment of larval abdominal motoneurons (Choi et al. 2004). Shaker is predominantly expressed in the presynaptic terminal and contributes to I\(_{Av}\) currents in the larval neuromuscular junction (Jan et al. 1977; Ganetzky and Wu, 1982; Wu et al. 1983, Singh and Wu 1990).

Mutations in eag also reduce I\(_{Av}\) in larval muscle, where synergistic effects of Shaker and
EAG have been observed (Ganetzky and Wu, 1982). Therefore, EAG contributes to $I_{\text{Av}}$ in the soma (our results) and in the presynaptic terminal.

**Heteromultimeric channel assembly**

The diversity of effects may reflect interactions of EAG with other channel subunits. It is unclear if EAG forms a homomeric channel or is capable of forming heteromultimers with other $K^+$ channel subunits. Evidence of EAG subunits interacting with other $K^+$ channels has been demonstrated previously. In *Xenopus* oocytes, Shaker and EAG channels interact functionally and this interaction is dependent on developmental time and expression levels (Chen et al. 2000; Tang et al. 1998). Hyperkinetic, a Shaker $\beta$ subunit homologue, and EAG show synergistic interactions in the *Drosophila* larval neuromuscular junction and also demonstrate a physical interaction (Wilson et al. 1998). Therefore, one possible reason for diverse potassium currents reduced in eag mutants and eag RNAi manipulations could be the heteromultimeric assembly of EAG with other $K^+$ channels. Due to our recording situation, the effects of EAG that we observed can be related to the somatodendritic regions of the neuron, but not the presynaptic terminals. In the soma of larval motoneurons, Shal seems to play a dominant role in contributing to $I_{\text{Av}}$ (Schaefer et al. 2010; Bergquist et al. 2010; Srinivasan et al. 2012). Since EAG also contributes to $I_{\text{Av}}$ (our results), we hypothesize that Shal and EAG could form heteromultimers in the somatic compartment. In the presynaptic terminal, Shaker and EAG contribute to $I_{\text{Av}}$. Therefore, Shaker and EAG could form heteromultimers in the presynaptic terminal. Apart from $I_{\text{Av}}$ current, $I_{Kv}$ is reduced in *eag sc29* and EAG RNAi experiments. Shal RNAi manipulations reduce $I_{\text{Av}}$ but...
not $I_{Kv}$, suggesting that the reduction in multiple $K^+$ currents is observed only in EAG manipulations, further supporting the formation of heteromultimers.

**Other possible roles of EAG**

We observed allele-specific reduction in $K^+$ currents, as documented elsewhere (Zhong and Wu 1991 and 1993). Multiple reasons may be given for this observation. First, the eag mutants used in the study could produce different partial or disrupted protein fragments (as demonstrated by Drysdale et al. 1991). Second, the C-terminus of EAG can be modulated by cyclic-nucleotides and CAMKII (Zhong and Wu 1993; Griffith et al. 1994; Wang et al. 2002; Sun et al. 2004). Direct modulation of EAG channels by cyclic-nucleotides has been demonstrated in oocytes (Bruggemann et al. 1993). Allele specific differences in modulation of $I_{AV}$ and $I_{Kv}$ currents by cGMP have also been observed in *Drosophila* larval muscles (Bhattacharya et al. 1999; Zhong and Wu 1993). Since we are performing *in-situ* whole-cell recordings it is possible that disruption of the membrane alters modulation of EAG channels during measurement of $K^+$ currents. Third, the reduction of EAG could result in upregulation of other $K^+$ channels, such as Shaker and Shal (Peng and Wu 2007). Increased expression of a Shal homologue, Kv4, in the lobster stomatogastric ganglion leads to an increase in hyperpolarization-activated cyclic nucleotide-gated channels (Maclean et al. 2003). In *Drosophila* larvae, reduction of Shal leads to upregulation of Shaker (Bergquist et al. 2010).

We demonstrate an embryonic requirement of EAG. $I_{Kv}$ was significantly reduced in chronic eag RNAi knock down compared to larval knockdown, suggesting that the
expression of these EAG currents is required throughout embryonic and larval stages. By contrast, \( I_{Av} \) was reduced in both chronic and larval manipulations, suggesting contribution of EAG to the current in third instar motoneurons. Interestingly, the total transient current was reduced by larval, but not chronic eagRNAi expression, suggesting that compensation may have occurred in the latter case. In fact no reduction in \( I_{A(Ca)} \) was observed in \( eag^{x-6} \) or in temperature manipulations, but an upregulation was observed in non-conditional eag RNAi. It is possible that \( I_{A(Ca)} \) is upregulated to compensate for the reduction observed in \( I_{Av} \). If such compensations occurred, however, it did not restore normal firing, since we observed the spontaneous events and reduced delay to spike following EAG knockdown.

In our experiments we observe discrepancies in the reduction of \( K^+ \) currents in different EAG manipulations. \( eag^{sc29} \) reduced total transient \( I_{Av} \) and \( I_{A(Ca)} \) currents. By contrast, in non-conditional EAG RNAi and chronic temperature sensitive EAG manipulations, which should mimic \( eag^{sc29} \) and \( eag^{x-6} \), only \( I_{Av} \) current is reduced, but not total transient and \( I_{A(Ca)} \). Such variability in knockdown has been observed among eag mutants in the larval muscle, and suggests that multiple \( K^+ \) currents are influenced by EAG (Zhong and Wu 1991). Similarly in non-conditional EAG RNAi, no reduction in total sustained current or \( I_{K(Ca)} \) was observed, whereas in chronic EAG manipulations, total sustained \( K^+ \) current was reduced.

**EAG influences excitability in motoneurons by altering firing properties**

Variable physiological phenotypes were observed in \( eag \) mutants. Large spontaneous spike-like events were often observed in mutants with little or no current injection. These
events may reflect spontaneous action potentials invading the cell body from the spike initiating region and/or action potentials evoked by large EPSPs. This phenotype could, therefore, result from both pre- and postsynaptic absence of EAG. To distinguish these effects, eag RNAi was expressed in all neurons to mimic eag mutants. Large spontaneous events were also observed in ELAV::eag RNAi. EAG is found in the synaptic neuropil and is absent in eag sc29 (Sun et al. 2004). Thus, similar to eag mutants, ELAV::eag RNAi manipulation could also result in increased synaptic drive to motoneurons. Spontaneous events were not observed, however, when eag RNAi was expressed in motoneurons only, further suggesting the contribution of increased synaptic drive. It should be noted, however, that ELAV-GAL4 is a stronger driver than RRA-GAL4. Therefore, the differences observed between the two eag RNAi manipulations could reflect differences in the degree of knockdown.

Hyperpolarizing the cell increased the amplitude of spontaneous events, consistent with increased driving force for both EPSPs and action potentials, but the reduction in frequency suggests the involvement of voltage-gated currents in the postsynaptic motoneuron. Although RRA-GAL4 manipulation did not cause spontaneous spiking, large EPSPs were sometimes observed and there were clear effects on K⁺ currents. A reduction in A-type K⁺ current in the dendritic region could be responsible for the large EPSPs. On the other hand, a decreased delay to the first spike was observed in eag mutants, suggesting reduction in the density of K⁺ channels in the spike-initiating zone. aCC motoneurons in eag mutants also have a more depolarized resting membrane potential than wild type.
Thus, the different phenotypes observed following EAG manipulations could reflect changes in the density and distribution of EAG in various regions of the neuron. The C-terminus of EAG has been shown to translocate to the nucleus (Sun et al. 2009), which may produce pleiotropic effects. Therefore, the variability observed in the firing phenotypes and K⁺ currents could also reflect downstream regulation of transcription by EAG (and see below). EAG performs multiple roles in regulating excitability in the cell, therefore the interpretation of the manifested phenotypes in relationship to specific mechanistic roles becomes challenging.

EAG senses and regulates neuronal excitability

Given the wealth of information regarding the differential functions of EAG, it is possible that EAG could act as a sensor of excitability. Several lines of evidence support such speculation. The protein structure of EAG suggests it is an amalgam of inward rectifier, depolarization-activated and cyclic nucleotide-gated channels. The C terminus of EAG has numerous sites for modulation. Synthesis of a non-ion conducting EAG80 splice variant is triggered by calcium influx and by activation of PKA or PKC. Translocation of EAG80 to the nucleus provides yet another interesting link between transcriptional regulation and excitability (Sun et al. 2009). Another example is provided by the translocation of C terminal fragment of Cav1.2 channels to the nucleus. Similar to EAG, intracellular calcium triggers the synthesis of Calcium Channel Associated Transcription regulator (CCAT), which regulates transcription (Gomez-Opsina et al. 2006). Sensory neurons in the mouse vomeronasal organ showed stimulus dependent regulation of Ether-a-go-go related gene expression (ERG) (Hagendorf et al. 2009).
Depending upon the sensory stimulus, ERG expression levels and resultant K⁺ current were altered, providing the first *in vivo* evidence for EAG related genes as sensors of excitability (Hagendorf et al. 2009). We provide evidence that EAG regulates excitability and firing patterns in *Drosophila* larval motoneurons, and contributes to multiple K⁺ currents in T1aCC. The variability in firing phenotypes we report in *eag* mutants is consistent with the possibility that EAG subunits could co-assemble with diverse K⁺ channels, depending upon the state of excitability of a neuron.

As demonstrated in this study, EAG represents a class of ion channels that performs multiple roles to regulate excitability. Unlike other K⁺ channels, mutations in EAG do not cause a single defect, but produce variable phenotypes. Participation of EAG in signaling mechanisms exacerbates these phenotypes. Translocation of EAG to nucleus provides yet another avenue for translating changes in excitability to gene expression. Studies in oocytes and cell culture systems cannot fully test the potential function of EAG, since these systems lack the complete gamut of proteins, signaling systems, and external stimulus that neurons experience. It will be worthwhile to test these possibilities using high resolution *in vivo* imaging to monitor localization and translocation of EAG.

**Acknowledgements:** The authors would like to thank the generous donation of *eag* mutants from Dr. B. Ganetzky. This study was supported by NIH grant NS28495 to RBL.
References:


Figure legends

Figure 1

EAG contributes to voltage-activated transient K+ current

A & B) Voltage-clamp records from CS, eag<sup>x-6</sup> and eag<sup>sc29</sup>. Cells were held at -80mV and 20mV, 200ms command steps ranging from -120mV up to 60mV were provided in TTX containing Ca++-A external solution. Leak subtraction was performed in all recordings. A) Total K+ currents in T1aCC. Peak transient K+ currents were clearly visible from -20mV. C) Graph of command potential vs. peak transient K+ current density in CS, eag<sup>x-6</sup> and eag<sup>sc29</sup>. Total transient K+ current was reduced in eag<sup>sc29</sup> but not in eag<sup>x-6</sup>. B) Voltage-activated I<sub>Av</sub> was isolated using cadmium chloride to block Ca++ channels from the same cell using the same voltage-clamp protocol. D) Graph of command potential vs. I<sub>Av</sub> current density in CS, eag<sup>x-6</sup> and eag<sup>sc29</sup>. I<sub>Av</sub> was significantly reduced in eag<sup>sc29</sup> but only at 40 and 60mV in eag<sup>x-6</sup>. E) Graph of command potential vs. I<sub>A(Ca)</sub> current density in CS, eag<sup>x-6</sup> and eag<sup>sc29</sup>. I<sub>A(Ca)</sub> was measured by subtracting I<sub>Av</sub> current in Fig. 1B from total transient K+ current in Fig. 1A. I<sub>A(Ca)</sub> was reduced in eag<sup>sc29</sup> from 20 up to 60mV but not in eag<sup>x-6</sup>. Two-way ANOVA followed by Bonferroni post-test were used to recognize significance, *** indicates p<0.001. Scale bar: 500pA, 10ms.
Figure 2

**EAG contributes to voltage-dependent sustained K⁺ currents**

A & B) Voltage-clamp records from CS, *eag x-6* and *eag sc29*. Cells were held at -20mV holding potential and 20mV command steps were provided to obtain voltage-dependent sustained K⁺ currents. Leak subtraction was performed in all recordings. A) Total sustained K⁺ currents were clearly visible from 0mV. C) Graph of command potential vs. sustained K⁺ current density in CS, *eag x-6* and *eag sc29*. Total sustained K⁺ current was reduced in *eag sc29* but not in *eag x-6*. B) Voltage-activated sustained K⁺ current (I_{Kv}) was isolated using cadmium chloride to block Ca⁺⁺ channels from the same cell. D) Graph of command potential vs. I_{Kv} in CS, *eag x-6* and *eag sc29*. I_{Kv} was reduced in *eag sc29* but not in *eag x-6*. **Note:** A & B are representative voltage-clamp records obtained from a -20mV holding potential to visualize sustained K⁺ currents. Sustained K⁺ current measurements (C & D) were made from the same cells held at -80mV holding potential. E) Graph of command potential vs. I_{K(Ca)} current density in CS, *eag x-6* and *eag sc29*. I_{K(Ca)} was measured by subtracting I_{Kv} current in Fig. 2B from total sustained K⁺ current in Fig. 2A. A significant reduction in I_{K(Ca)} in *eag sc29* was observed at 40mV, but not in *eag x-6*. Two-way ANOVA followed by Bonferroni post-test were used to recognize significance, *** indicates p<0.001 comparison between CS and *eag sc29*. Scale bar: 500pA, 10ms.

Figure 3

**Targeted reduction of EAG in motoneurons**
A) Graph of total transient K\(^+\) current densities in RRA-GAL4 (control) and EAG knock down. Total transient K\(^+\) current was similar in eag RNAi manipulations compared to control. B) Graph of total sustained K\(^+\) current densities in RRA-GAL4 and EAG knock down. No reduction was observed. C) Graph of I\(_{A_v}\) in RRA-GAL4 and EAG knock down. I\(_{A_v}\) was significantly reduced in EAG manipulation compared to control. D) Graph of I\(_{K_v}\) in RRA-GAL4 and EAG knock down. I\(_{K_v}\) was significantly reduced only in the EAG manipulation. E) I\(_{A_{Ca}}\) was upregulated in EAG manipulation, whereas I\(_{K_{Ca}}\) was unchanged (F). * indicates p<0.05. Confocal images of single RP2 motoneurons in third instar larval CNS. G) Control larval RP2 cell with normal dendrites H) RP2 cell expressing EAG RNAi. Note that there are no gross differences apparent between the dendritic structures of the motoneuron in control and EAG-RNAi individuals. Detailed description of the genotypes is provided in the methods section. Scale bar denotes 20 \(\mu\)M.

**Figure 4**

**Larval and chronic knock down of EAG**

Temperature-sensitive GAL80 was used to reduce EAG levels in T1aCC. Temperature shift paradigm is shown in the top right corner. Chronic indicates eag RNAi knock down from embryo (E) to wandering third instar larval stage (WL3). L3 indicates eag RNAi knock down from mid (M) third instar larvae until wandering third instar, when recordings were performed. Parental control larvae were raised in the L3 temperature shift paradigm. A) Temperature-sensitive GAL80 blocks GAL4 expression leading to no expression of GFP in dorsomedial motoneurons at 18°C. B) GAL80 is blocked at higher
temperatures (30°C) leading to expression of GFP in dorsomedial motoneurons. Scale bar denotes 20 μM. C) Graph of total transient K⁺ current densities in control, chronic and L3 knock down. Total transient K⁺ current was significantly reduced in the larval eag RNAi manipulations compared to control. D) Graph of total sustained K⁺ current densities in control, chronic and L3 knock down. Both chronic and L3 eag RNAi knock down showed a significant reduction compared to control. E) Graph of Iₐᵥ in control, chronic and L3 knock down. Iₐᵥ was significantly reduced in both L3 and chronic manipulations compared to control. F) Graph of Iₖᵥ in control, chronic and L3 knock down. Iₖᵥ was reduced only in the chronic manipulation at 60mV compared to the L3 eag RNAi knock down. No change was observed between control and chronic knock down. Two-way ANOVA followed by Bonferroni post-test were used to recognize significance, *** indicates p<0.001, ** indicates p<0.01 and * indicates p<0.05 comparison between control and knock down. ### indicates p<0.001, comparison between chronic and L3 eag RNAi knock down.

Figure 5

Influence of eag mutants and eag RNAi manipulations on firing properties

Representative current-clamp recordings from A) CS, B) eag⁻⁶ and C) eag⁻⁶⁹ D) RRA-GAL4, E) ELAV::eag RNAi and F) RRA::eag RNAi in T1aCC from a normalized membrane potential of -60mV. Dotted black box in B, C and E denotes spontaneous spike-like events, absent in CS (A). Black box in F denotes example of large EPSPs. G and H) Firing frequency plots of eag mutants and eag RNAi manipulations in
comparison with CS and RRA. Evoked firing was comparable in all genotypes.

Spontaneous spike-like events were observed in eag<sup>x-6</sup> and eag<sup>sc29</sup> and ELAV::eag RNAi but not in CS, RRA and RRA::eag RNAi.

Figure 6

Influence of synaptic input in CS and eag<sup>x-6</sup>

A) Extended periods of rhythmic motoneuron activity in CS and eag<sup>x-6</sup>. Black box denotes rhythmic synaptic input (expanded time scale in line 2, black arrow) and dotted black box denotes baseline synaptic activity (highlighted in the dotted black box) in both CS and eag<sup>x-6</sup>. Black bar denotes -60mV in both sets of traces. B) Bias current was injected to hyperpolarize the membrane in eag<sup>x-6</sup> to observe the effects of membrane potential on spontaneous spike-like events. At -20mV action potentials with clear after-spike hyperpolarization were observed. At -60mV spontaneous spike-like events (absent in CS) were observed with no visible after-spike hyperpolarization. At -95mV these events became larger in amplitude but reduced in frequency.

Table 1 Firing properties

Summary of the resting membrane potential, input resistance (measured from voltage-clamp experiments) and frequency of physiological phenotypes in various genotypes tested. * denotes comparison between ELAV::eag RNAi and RRA, p<0.05 and # denotes comparison between mutants and CS. Student t-test was used to recognize significance. ** indicates examples (dotted black box) in Fig. 5 and ## indicates examples (black box)
Table 2

Summary of effects of eag manipulations on $K^+$ currents

Comparison of the effect of eag manipulations in total transient, total sustained $K^+$ current, $I_{Av}$, $I_{Kv}$, $I_{A(Ca)}$ and $I_{K(Ca)}$. Note, reduction indicates results from the post-test performed on data described in figures 1, 2 and 3 and 4. NC: indicates non-conditional eag RNAi experiments in Fig. 3. C: indicates temperature sensitive “conditional” experiments in Fig. 4.
A B

Total sustained K⁺ current

CS

-eag x-6

-eag sc29

-20mV

Current (pA/pF)

Command potential (mV)

E

Ik(Ca)

CS

eag x-6

eag sc29

-120 -100 -80 -60 -40 -20 0 20 40 60

0 10 20 30 40 50 60

C D

I_{kv}

CS

-eag x-6

-eag sc29

-20mV

Current (pA/pF)

Command potential (mV)
A. Total transient $K^+$ current

B. Total sustained $K^+$ current

C. $I_{av}$

D. $I_{kv}$

E. $I_{A(Ca)}$

F. $I_{K(Ca)}$

G. Total transient $K^+$ current

H. Total sustained $K^+$ current

[Images and graphs showing current-voltage relationships for different currents and conditions]
A. Control larvae raised at 18°C
B. Temperature raised to 30°C

C. Total transient K⁺ current
D. Total sustained K⁺ current

E. $I_{AV}$
F. $I_{KV}$
Table 1

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Resting membrane potential (mV)</th>
<th>Input resistance (MΩ)</th>
<th>Normal firing</th>
<th>Increased amplitude of EPSPs ##</th>
<th>Spontaneous events **</th>
</tr>
</thead>
<tbody>
<tr>
<td>CS (8)</td>
<td>-49.2±1 #</td>
<td>383.16 ±49</td>
<td>8/8</td>
<td>0/8</td>
<td>0/8</td>
</tr>
<tr>
<td>eag x-6 (10)</td>
<td>-34.5±2 #</td>
<td>396.73±88</td>
<td>1/10</td>
<td>5/10</td>
<td>3/10</td>
</tr>
<tr>
<td>eag sc29 (16)</td>
<td>-37.7±1</td>
<td>378.79±10</td>
<td>7/16</td>
<td>3/16</td>
<td>4/16</td>
</tr>
<tr>
<td>RRA (7)</td>
<td>-45.1±1</td>
<td>345.32±19</td>
<td>7/7</td>
<td>0/8</td>
<td>0/8</td>
</tr>
<tr>
<td>RRA:: eag RNAi (10)</td>
<td>-42.9±3</td>
<td>409.54±19</td>
<td>7/10</td>
<td>2/10</td>
<td>0/8</td>
</tr>
<tr>
<td>ELAV::eag RNAi (9)</td>
<td>-37±3*</td>
<td>286.93±8</td>
<td>5/9</td>
<td>0/9</td>
<td>3/9</td>
</tr>
</tbody>
</table>

Table 2

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Total transient K⁺ current</th>
<th>(I_{av})</th>
<th>Total sustained K⁺ current</th>
<th>(I_{kv})</th>
<th>(I_{A(Ca)})</th>
<th>(I_{K(Ca)})</th>
</tr>
</thead>
<tbody>
<tr>
<td>eag x-6 (compared to CS)</td>
<td>No change</td>
<td>Reduced</td>
<td>No change</td>
<td>No change</td>
<td>No change</td>
<td>Increased</td>
</tr>
<tr>
<td>eag sc29 (compared to CS)</td>
<td>Reduced</td>
<td>Reduced</td>
<td>Reduced</td>
<td>Reduced</td>
<td>Reduced</td>
<td>Reduced</td>
</tr>
<tr>
<td>NC: eag RNAi (compared to RRA)</td>
<td>No change</td>
<td>Reduced</td>
<td>No change</td>
<td>Reduced</td>
<td>Increased</td>
<td>No change</td>
</tr>
<tr>
<td>C: L3 eag RNAi (compared to Control)</td>
<td>Reduced</td>
<td>Reduced</td>
<td>Reduced</td>
<td>No change</td>
<td>No change</td>
<td>Reduced</td>
</tr>
<tr>
<td>C: chronic eag RNAi (compared to Control)</td>
<td>No change</td>
<td>Reduced</td>
<td>No change</td>
<td>Reduced</td>
<td>No change</td>
<td>Reduced</td>
</tr>
<tr>
<td>C: Chronic Vs late larval eag RNAi</td>
<td>Reduced</td>
<td>No change</td>
<td>No change</td>
<td>Reduced</td>
<td>No change</td>
<td>Reduced</td>
</tr>
</tbody>
</table>