1 The *Drosophila* ERG channel *seizure* plays a role in the neuronal homeostatic stress response

2 Short title: ERG channels are required for neuronal homeostasis

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Abstract

14 Neuronal physiology is particularly sensitive to acute stressors that affect excitability, many of

15 which can trigger seizures and epilepsies. Although intrinsic neuronal homeostasis plays an

16 important role in maintaining overall nervous system robustness and its resistance to stressors,

17 the specific genetic and molecular mechanisms that underlie these processes are not well

18 understood. Here we used a reverse genetic approach in *Drosophila* to test the hypothesis that

19 specific voltage-gated ion channels contribute to neuronal homeostasis, robustness, and stress

20 resistance. We found that the activity of the voltage-gated potassium channel *seizure* (*sei*), an

21 ortholog of the mammalian ERG channel family, is essential for protecting flies from acute heat-

22 induced seizures. Although *sei* is broadly expressed in the nervous system, our data indicate

23 that its impact on the organismal robustness to acute environmental stress is primarily

24 mediated via its action in excitatory neurons, the octopaminergic system, as well as glia.

25 Furthermore, our studies suggest that human mutations in the human ERG channel (hERG),

26 which have been primarily implicated in the cardiac Long QT Syndrome (LQTS), may also

27 contribute to the high incidence of seizures in LQTS patients via a cardiovascular-independent

28 neurogenic pathway.

29

Author Summary

31 Neurons are extremely sensitive to diverse environmental stressors, including rapid changes in

32 the ambient temperature. To buffer stress, all animals have evolved diverse physiological

33 mechanisms to protect neuronal activity from acute and chronic stressors, and failures of these

34   safeguards often lead to hyperexcitability, episodic seizures, and chronic epilepsy. Although

35   seizures and related syndromes are common, their underlying molecular and genetic factors,

36   and their interactions with environmental triggers, remain mostly unknown. Here, we show

37   that in the fruit fly, mutations in the ERG voltage-gated potassium channel *seizure* (*sei*), an

38   ortholog of the human hERG channel that has been previously implicated in the cardiac Long-

39   QT syndrome, could also increase seizure susceptibility. We demonstrate that in addition to its

40   cardiac expression, the *sei* channel is broadly expressed in the nervous system, specifically

41   localized to axonal projections, and is specifically required in excitatory and modulatory

42   neurons, as well as non-neuronal glia for maintaining organismal resistance to heat-induced

43   seizures. Thus, our work suggests that the previously reported increase in seizure susceptibility

44   in individuals with mutations in hERG are likely directly related to its neuronal action,

45   independent of its cardiac function.

46   **Introduction**

47   Neuronal homeostatic responses to acute and long-term environmental stressors are essential

48   for maintaining robust behavioral outputs and overall organismal fitness [1-3]. At the neuronal

49   level, the homeostatic response to stress depends on both synaptic and cell-intrinsic

50   physiological processes that enable neurons to stably maintain optimal activity patterns [4-6].

51   Previous theoretical and empirical studies have suggested that the neuronal intrinsic

52   robustness depends on the expression and activity of specific combinations of ion channels and

53   transporters, which can vary across neuronal cell types and individuals [7-10]. While some of

54   the transcriptional and physiological processes that enable neurons to adjust their intrinsic

55   activity levels in response to long-term stressors have been identified [11-13], most of the

56   genetic and molecular mechanisms that mediate susceptibility to acute, environmentally-

57   induced seizures, such as fever-induced febrile seizures, remain unknown [14-16].

58   Because of its small size, large surface-to-volume ratio, and its inability to internally regulate

59   body temperature, the fruit fly *Drosophila melanogaster*, represents an excellent model for

60   studying mechanisms underlying the neuronal acute response to heat stress, which typically

61   leads to seizure-like behavior, followed by paralysis [17-20]. Here we utilized this model to test

62   the hypothesis that the knockdown of genes that are specifically important for the intrinsic

63   neurophysiological homeostatic response to acute heat stress, would have little impact on fly

64   behavior at permissive temperatures, but would lead to rapid paralysis under acute heat-stress

65   conditions.

66   To test our hypothesis, we first employed a reverse genetic approach to identify candidate

67   genes specifically involved in the neuronal homeostatic response to acute heat stress. By using

68   a tissue-specific RNAi knockdown screen of voltage-gated potassium channels, we identified

69   *seizure* (*sei*), the fly ortholog of the mammalian hERG channel (KCNH2) [18, 21-25], as an

70   essential element in the neuronal homeostatic response to acute heat stress. Previous studies

71   have indicated that dominant hERG mutations, which are one of the primary genetic causes for

72   the cardiac Long QT Syndrome (LQTS) in humans [26, 27], are also associated with a high

73   prevalence of generalized seizures [28]. Yet, it is currently assumed that seizures in LQTS

74   patients represent a derived secondary outcome of the primary cardiac pathology [29-31].

75   However, the data presented here, as well as previous studies that showed that ERG channels

76   are expressed in mammalian neuronal tissues [32, 33], and contribute to intrinsic spike

77   frequency adaptation in cultured mouse neuroblastoma cells and cerebellar Purkinje neurons

78   [34, 35], suggest that ERG channels also have a specific function within the nervous system.

79   By utilizing existing and novel genetic tools, here we show that the ERG channel *sei* is indeed

80   essential for maintaining neuronal robustness under acute heat stress conditions in *Drosophila*.

81   Specifically, we demonstrate that although *sei* is broadly expressed in the nervous system of

82   the fly, its contribution to the organismal behavioral resistance to acute heat stress is primarily

83   mediated via its specific action in excitatory cholinergic and glutamatergic neurons, the

84   octopaminergic system, as well as non-neuronal glia. Furthermore, by generating a

85   CRISPR/cas9-derived GFP-tagged allele of the native *sei* locus, we also show that at the

86   subcellular level, *sei* exerts its action primarily in neuronal axons. Together, these studies

87   indicate that mutations in hERG-like potassium channels may contribute directly to the etiology

88   of stress-induced seizures in susceptible individuals by limiting the intrinsic neuronal

89   homeostatic response to acute environmental stressors, possibly via homeostatic axonal spike

90   frequency adaptation.

91

92   **Results**

93   **Neuronal *sei* gene knockdown leads to acute heat-induced seizures**

94   Previously published theoretical models and empirical studies have indicated that the actions of

95   diverse voltage-gated potassium channels mediate action potential repolarization, and

96   modulate the action potential threshold [36-39], which are important for the homeostatic

97   regulation of synaptic activity and excitability [40-42]. Yet, which genes regulate the intrinsic

98   capacity of neurons to buffer environmentally-induced hyperexcitability is mostly unknown.

99   Thus, we initially hypothesized that the intrinsic ability of neurons to buffer acute heat stress is

100   mediated, at least in part, by the action of specific voltage-gated potassium channels. Because

101   most of these channels are expressed in both neuronal and non-neuronal tissues, we tested our

102   hypothesis by using a neuronal-specific RNAi-dependent knockdown screen of all genes that

103   encode voltage-gated potassium channels in the *Drosophila* genome. This screen revealed that

104   neuronal knockdown of the gene *seizure* (*sei*), which encodes the sole fly ortholog of hERG-like

105   voltage-gated potassium channels [23, 24], has the strongest effect on lowering the threshold

106   to heat-induced seizures (Fig 1A-B). Therefore, we focused our following primary working

107   hypotheses on the contribution of the *sei* channel to the intrinsic neuronal homeostatic

108   response to stress.

109   Mutations in the *seizure* (*sei*) gene were initially identified in a forward genetic screen for

110   temperature-sensitive (ts) alleles of excitability-related genes in *Drosophila* [23, 24]. Because *sei*

111   was assumed to be an essential component of general neuronal excitability, the two original

112   EMS-induced *sei* mutant alleles were assumed to be structural ts alleles [18, 23-25]. However,

113   because recent studies indicate that these *sei* alleles are more likely null or hypomorphic [25],

114   we hypothesized that the action of the *sei* channel is specifically required for the ability of

115   neurons to respond to acute heat stress. Thus, we next used a null allele of *sei* [43, 44] to

116   demonstrate that *sei* activity is specifically required for the ability of adult flies to resist the

117   impact of acute heat stress (Fig 2A-B). In addition to its role in the neuronal response to acute

118   heat stress, we also show that the *sei* mutation impaired the ability of flies to adapt to a gradual

119   heat stress (Fig 2C-D). Together, these data indicate that *sei* activity is not required for basal

120   neuronal excitability, but is essential for the ability of neurons to maintain stable and adaptive

121   firing rates under fluctuating environmental conditions.

122   In contrast to adult flies, which under natural conditions could easily escape stressful

123   environmental conditions by flying, larvae are much more constrained. Thus, the protective role

124   of *sei* might be more ecologically relevant to the pre-adult developmental stages. Our data

125   indicate that, as in the adult, *sei* activity is also necessary for normal larval locomotion under

126   acute heat stress conditions (Fig 2E). Furthermore, because larvae can sense acute nociceptive

127   stimuli, such as heat, via the activity of their cuticular multidendritic (md) sensory neurons [45-

128   47], we next tested the hypothesis that heat induced hyperexcitability of md neurons in *sei*

129   mutants would lead to nociception hypersensitivity. Indeed, we found that *sei* mutant larvae

130   exhibit a significantly faster response to heat stimuli relative to wild type control (Fig 2F), which

131   suggests that their nociceptive system is hypersensitive. Together, these studies indicate that

132   the *sei* channel plays an important role in maintaining neuronal stability and robustness, and

133   protecting *Drosophila* neurons from environmentally-induced hyperexcitability.

134   Because previous investigations of the impact of temperature changes on neuronal activity

135   have shown that neurons will respectively increase or decrease their firing rates in response to

136   a rise or fall in ambient temperature [48-50], we next hypothesized that *sei* mutant flies might

137   be protected from the effect of acute cold stress on neuronal activity. However, we found no

138   effect of the *sei* mutation on larval locomotion at 13°C relative to wild type controls (Fig 2H).

139   Thus, although the precise biophysical role of hERG-type voltage-gated potassium channels in

140   regulating neuronal excitability remains elusive, the *in vivo* data presented here, as well as

141   previously published *in vitro* studies [34, 35], indicate that hERG channels play a specific role in

142   maintaining optimal neuronal activity by protecting neurons from environmentally-induced

143   hyperexcitability but not hypoexcitability [25].

144

145   **Organismal resilience to heat stress requires the action of *sei* in excitatory and**

146   **octopaminergic neurons, as well as glia**

147   The *sei* gene is expressed in diverse neuronal and non-neuronal cell types, including cardiac and

148   muscle cells [32, 51]. Previous studies by us and others have shown that mutations in *sei*

149   increase the overall organismal sensitivity to acute heat stress [23-25]. Yet, whether this

150   organismal phenotype is driven by the action of *sei* in all cell types that express it was unknown.

151   Therefore, we next used tissue-specific RNAi knockdown to determine which cell types require

152   *sei* activity to protect animals from heat-induced seizures. Similarly to our previous work [25],

153   we found that neuronal-specific knockdown of *sei* is sufficient to phenocopy the effect of the

154   null allele on the susceptibility of adult flies to heat-induced seizures (Fig 1A-B, 3A-B). However,

155   we were also surprised to find that, although not as striking as in the neuronal knockdown, the

156   organismal response to heat stress also depends on the activity of *sei* in non-neuronal glia (Fig

157   3C-D), but not in the heart or muscles (Fig 3E-H). Together, these data suggest that the

158   organismal resistance to acute heat-induced seizures requires *sei* activity in the two primary cell

159   types of the nervous system, neurons and glia.

160   Because the fly CNS is a compact mosaic of different cell types, it is hard to establish whether

161   *sei* is primarily expressed in neurons or glia. To address this, we generated a transgenic

162   *Drosophila* driver line that expresses the *LexA* activator under the control of the putative *sei*

163   promoter sequences [52, 53]. We then used this line to express a nuclear EGFP reporter, which

164   indicated that *sei* is broadly expressed throughout the central nervous system (Fig 4A-B). To

165   specifically identify *sei*-expressing glia, we next combined the *sei*-LexA line driving a nuclear

166   EGFP with the glia-specific *Repo-GAL4* line driving a nuclear *RFP* (*RedStinger*). Confocal imaging

167   of co-labeled brains showed that in addition to its broad neuronal expression pattern, *sei* is also

168   expressed in a small fraction of brain glia (Fig 4C-E, arrows). These data further indicate that the

169   action of *sei* in the fly CNS is primarily mediated by its action in most neurons and few glia.

170   Neurons are comprised of diverse cell types with different physiological properties and varying

171   contributions to systems-level neural excitability. Therefore, we next wished to determine

172   which neuronal subtypes might require *sei* activity for enabling the organismal response to

173   acute heat stress. A broad screen of *sei* knockdown using several neuronal type-specific *GAL4*

174   driver lines revealed that the organismal response to heat stress depends on the expression of

175   *sei* in cholinergic (Fig 5A-B) and glutamatergic (Fig 5C-D) excitatory neurons, but not in

176   GABAergic inhibitory neurons (Fig 5E-F). We also observed a significant effect of *sei* knockdown

177   in the modulatory octopaminergic system (Fig 5G-H) but not in the dopaminergic, serotonergic,

178   peptidergic, or the peripheral sensory systems (Fig 5I-P). Thus, our data suggest that *sei* plays

179   an important role in protecting the nervous system from environmental stressors that could

180   lead to general hyperexcitability and seizures by maintaining the neuronal robustness of

181   excitatory and some neuromodulatory neurons.

182

183   **SEI channels are localized to axonal processes**

184   The subcellular localization of various voltage gated ion channels plays an important role in

185   determining how they might be contributing to neuronal signaling and excitability [54-56]. For

186   example, ion channels localized to axons generally impact action potential generation,

187   propagation, and modulation, while those localized to dendrites influence integration of

188   synaptic inputs, propagation of electrical activity to the soma, and action potential

189   backpropagation [54, 57, 58]. Nevertheless, ion channels with specific subcellular enrichment in

190   either dendrites, cell bodies, axons, or presynaptic terminals have all been implicated in human

191   epilepsies [59]. Therefore, we next determined the subcellular localization of native SEI

192   channels by generating a C-terminus GFP-tagged allele of the endogenous *sei* locus (Fig 6A). We

193   found that the response of homozygous *seiGFP* flies to heat stress is not different from wild

194   type animals, which indicates that the tagged protein forms wild-type like channels (Fig 6B-C).

195   We next used an anti-GFP antibody to probe the subcellular spatial distribution of *seiGFP*

196   channels in the larval and adult nervous systems. These studies revealed that SEI is primarily

197   localized to the axonal membranes of most neurons, but not in somas or dendrites (Fig 7). The

198   enrichment of SEI channels in axons supports a model whereby ERG channels contribute to the

199   intrinsic homeostatic regulation of optimal neuronal activity via the modulation of action

200   potentials, a model further supported by the previously reported influence of mammalian ERG

201   channels on spike frequency adaptation in cultured mammalian neurons [34, 35].

202

203   **Discussion**

204   Previous theoretical and empirical studies of neural circuit adaptability, and by extension, the

205   ability of animals to maintain robust and adaptive behavioral outputs in unstable environments,

206   depends on both the intrinsic homeostatic capacity of neurons to maintain an optimal activity

207   pattern, and the ability of neural circuits to maintain stable outputs via the homeostatic

208   regulation of neuronal connectivity and synaptic activity [4-6]. Yet, despite its high incidence,

209   the majority of genetic and molecular factors that regulate neuronal homeostasis, and increase

210   susceptibility to seizures, remain mostly unknown [14-16]. Here, we show that the *Drosophila*

211   voltage-gated potassium channel *sei*, a conserved ortholog of the human hERG gene, is

212   required for the neuronal homeostatic response to acute heat stress. Furthermore, we show

213   that the organismal capacity to buffer the effects of acute heat stress depends on the

214   independent activity of *sei* in both neurons and glia. We also found that although *sei* is broadly

215   expressed in the nervous system, its contribution to the overall organismal resistance to acute

216   heat stress seems to be specifically driven by its action in cholinergic and glutamatergic

217   excitatory neurons, as well as to a lesser extent in the modulatory octopaminergic system. In

218   addition, we developed genetic tools to show that SEI is expressed in the axons of neurons and

219   in glia. Together, our data highlight the important role of *sei* in the organismal homeostatic

220   response to acute environmental stress, by providing robustness to both the intrinsic activity of

221   specific neuronal populations, and the neural circuits that harbor them.

222   How ERG-type potassium channels might contribute to neuronal intrinsic homeostasis during

223   bouts of acute stress is not well understood. Nonetheless, *in vivo* and *in vitro* studies in

224   *Drosophila* and mammalian models have suggested that ERG channels have little effect on

225   baseline neuronal firing rate, but can prevent rapid firing in response to environmental or

226   electrophysiological stimuli that induce hyperexcitability [25, 34, 35]. We have previously

227   shown that in *Drosophila* motor neurons, basal neuronal firing patterns are unaffected by the

228   *sei* mutation at optimal 25°C, but become hyperexcitable in response to a rapid temperature

229   increase [25]. Similarly, in electrophysiological studies of mammalian brain slices, *in vitro*

230   cultured neurons, and heterologously-expressed mammalian ERG channels, pharmacological

231   blockers of hERG channels have little effect on firing rates in response to small current

232   injections, but greatly diminish spike frequency adaptation in response to large current

233   injections, resulting in rapid firing rates [34, 35]. The presence of SEI channels specifically in

234   axons (Fig 7) suggests that they do not affect the propagation of dendritic potentials, but rather

235   limit the rate of action potential generation and propagation, which is sufficient to prevent

236   rapid firing rates. Together, these data suggest a model whereby ERG-like potassium channels

237   play a crucial role in mediating the neuronal homeostatic response to acute stress by protecting

238   neurons from rapid increase in firing rates, and therefore, support neuronal robustness when

239   exposed to extreme environmental fluctuations.

240   At the neuronal network level, seizures are thought to result from an imbalance between

241   excitatory and inhibitory neural signaling pathways [15, 60]. We found that knocking down *sei*

242   specifically in all cholinergic neurons, the primary excitatory pathway in the fly central nervous

243   system, is sufficient to phenocopy the effects of *sei* null mutations on the organismal resistance

244   to heat-induced seizures. These results are similar to previous studies, which showed that

245   increasing activity of the cholinergic system in flies, via genetic manipulations of voltage gated

246   sodium channels and optogenetic neural activation, is sufficient to increase seizure-related and

247   paralytic behavior [61-64]. The simplest interpretation of these data together is that the lack of

248   *sei* in cholinergic excitatory neurons makes them hypersensitive to heat-induced

249   hyperexcitability, which subsequently surpasses the buffering capacity of the inhibitory

250   neurotransmission pathways, and therefore leads to the rapid development of generalized

251   seizures and paralysis.

252   Additionally, we observed a large increase in seizure susceptibility when *sei* is knocked down in

253   glutamatergic neurons. Although we and others have previously demonstrated that the activity

254   of motor neurons, which in insects are primarily glutamatergic, is increased in seizure-

255   susceptible mutant flies [61, 65], this finding suggests that the decreased intrinsic ability of

256   motor neurons to resist acute stress is sufficient for inducing organismal seizure-like phenotype

257   (Fig 5C-D). Nevertheless, we currently cannot exclude the possibility that the observed effect of

258   knocking down *sei* expression in glutamatergic neurons on organismal sensitivity to stress is

259   mediated via the action of a small number of modulatory glutamatergic neurons within the

260   central nervous system [66].

261   We also observed an impairment in the organismal homeostatic response to acute heat stress

262   when *sei* is specifically knocked-down in the modulatory octopaminergic system (Fig 5G-H).

263   Previous studies of the octopaminergic system in *Drosophila* and other insects have indicated

264   that octopamine and related biogenic amines have broad impact on diverse neuronal processes

265   at the developmental and physiological timescales [67, 68]. Because *sei* mutant flies seem to

266   have normal behaviors when housed under constant optimal conditions, it is likely that the

267   effects of knocking down *sei* in octopaminergic neurons on heat-induced seizures are

268   physiological, not developmental. Although we currently do not know which specific elements

269   of the octopaminergic system play a role in the organismal response to acute heat stress,

270   previous work has shown that exogenous application of octopamine in *Drosophila* increases

271   contraction force of muscles and their response to synaptically driven contractions [69].

272   Therefore, one possible mechanism by which *sei* knockdown in octopaminergic neurons might

273   affect observed heat-induced seizures is via the direct modulation of the neuromuscular

274   junction. Octopamine has also been shown to play important roles in the central nervous

275   system, including modulation of behaviors related to motivation, sleep, aggression, social

276   behaviors and learning and memory [67, 70-73]. Therefore, *sei* knockdown in octopaminergic

277   neurons may result in a broader shift in synaptic processes associated with the homeostatic

278   maintenance of the balance between excitatory and inhibitory pathways under acute stress

279   conditions.

280   The important role of *sei* activity in regulating the capacity of the nervous system to buffer

281   acute environmental stress is further supported by our discovery that its knockdown in glia also

282   increased susceptibility to acute heat-induced seizures (Fig 3C-D). Previous studies have

283   suggested that some glia cells are important for maintaining synaptic activity and homeostasis,

284   and that disrupting glia functions could contribute to the etiology of seizures because of their

285   role in modulating extracellular potassium concentration, adenosine levels, the size of the

286   extracellular space, and uptake of neurotransmitters [74-79]. Additionally, glia in the

287   mammalian hippocampus express muscarinic and nicotinic acetylcholine receptors [80-82], and

288   acetylcholine release in the hippocampus has been shown to increase GABAergic signaling

289   through activation of glial cells, possibly by glial release of glutamate [83]. If conserved, this

290   would be an intriguing mechanism whereby glia might control excitability throughout, or in

291   specific regions of, the *Drosophila* central nervous system. However, how the action of voltage

292   gated ion channels such as *sei* in glia might affect these specific processes remains mostly

293   unknown. Nevertheless, glial expression of another voltage gated potassium channel that is

294   associated with human epilepsy, KCNJ10, has been shown to lead to epileptic activity in a

295   mouse model, possibly via its role in buffering extracellular potassium and glutamate [84, 85].

296   Whether hERG-like channels play a similar role in glia remains to be explored.

297   Together, the data we present here provide important insights into the possible role of hERG

298   channels in regulating neuronal robustness and susceptibility to stress-induced seizures. From a

299   clinical perspective, our data suggest that the high incidence of generalized seizures that has

300   been reported in LQTS patients that carry mutations in the hERG genes [28] might not be a

301   secondary cardiogenic comorbidity, as is currently often assumed [29-31]. Instead, because

302   about 40% of patients that carry LQTS-related hERG mutations have reported a personal history

303   of seizures, as compared to less than 20% in LQTS patients with similar cardiac pathologies that

304   are due to mutations in other genes [28], we hypothesize that seizure etiology in many LQTS

305   patients is likely due to the direct impact of mutations in hERG on nervous system functions,

306   independent of their cardiovascular condition. Therefore, we predict that it is possible that

307   some unidentified mutations in hERG might be causally related to epilepsies, independent of

308   the presentation of any LQTS-related pathologies, and may represent novel genetic risk factors

309   for seizures.

310   The studies we describe here provide compelling evidence that hERG channels play an essential

311   role in protecting the nervous system from acute environmental stressors, such as heat, which

312   could potentially lead to hyperexcitability and seizures. Furthermore, we show that in

313   *Drosophila*, the activity of the ERG channel *sei* contributes to neuronal and behavioral

314   robustness via its action in independent cell types in the nervous system. These important

315   insights should help us to better understand how the nervous system responds to acute

316   environmental stressors, and possibly provide important mechanistic insights into some of the

317   known pathologies associated with hERG mutations in human patients.

318

319   **Materials and Methods**

320   **Fly Stocks and Genetics**

321   Flies (*Drosophila melanogaster*) were raised on standard corn syrup-soy food (Archon Scientific)

322   at 25°C temperature, 70% humidity, on a 12:12 light/dark cycle. Unless specifically noted, wild

323   type control line used was w^{1118}. All fly strains were either produced in the Ben-Shahar lab or

324   obtained from the Bloomington Stock Center (stock numbers in parentheses). UAS-RNAi TRiP

325   lines [86] used in the initial screen included *sei* (#31681), *shab* (#25805), *eag* (#31678), *shaker*

326   (#53347), *shaw* (#28346), *shal* (#31879), *elk* (#25821) and *kcnq* (#27252). The TRiP UAS-

327   *Luciferase* RNAi was used a control (#35789), and UAS-RNAi lines were driven by the *elav-GAL4;*

328   *UAS-Dicer2* line (#25750) (Fig 1A-B). For the cell-type-specific *sei* knockdown screen (Figs 3 and

329   5), the UAS-RNAi for *sei* and *luciferase* were each recombined with *UAS-Dcr2*. The original null

330   *seiP* allele from Bloomington (#21935) was backcrossed into the w^{1118} wild type strain. Other

331   transgenic lines from the Bloomington stock center included: *UAS-RedStinger* (#8546), *LexAOp-*

332   *GFPnls* (#29954), *elav-GAL4 [87]* (#458), *Repo-GAL4* (#7415), *hand-GAL4* (#48396), *ChAT-GAL4*

333   (#6798), *VGlut-GAL4* (#60312), *gad-GAL4* (#51630), *ple-GAL4* (#8848), *Tbh-GAL4* (#39939), *Trh-*

334   *GAL4* (#49258), and *C929-GAL4* (#25373). *BG57-GAL4* [88] and *PO163-GAL4* were from the

335   Dickman and Zlatic labs respectively.

336   The C-terminus GFP-tagged allele of *sei* was generated via CRISPR/*Cas9*-dependent editing by

337   using a modified "scarless" strategy (www.flyCRISPR.molbio.wisc.edu)[89, 90]. Specifically, four

338   sgRNAs TGTAAGCGAATACCACGTTG, GACAGCATTCTCCCGCAACG, GAAGCAGAAGCAGGTAACTC,

339   AGGTGAGTGAGTTACTCATC, which flank the targeted genomic *sei* sequence were designed

340   using flyRNAi.org/crispr. Complementary oligos that correspond to each individual sgRNA (IDT)

341   were cloned into the pDCC6 plasmid (a gift from Peter Duchek, Addgene plasmid # 59985),

342   which also includes the coding sequence for *Cas9* [91], by using the BbsI restriction enzyme

343   (NEB). The donor plasmid for homologous recombination was constructed by using a Golden

344   Gate assembly [92] to recombine four DNA elements: 1) A backbone with ampicillin resistance

345   (pBS-GGAC-ATGC plasmid (a gift from Frank Schnorrer, Addgene #60949)[93]; 2) Eye-specific

346   dsRed reporter driven by the 3XP3 promoter, flanked by two *PiggyBac* transposase recognition

347   sites, which was PCR-amplified from the pHD-ScalessDsRed plasmid (a gift from Kate O'Connor-

348   Giles, Drosophila Genome Resource Center #1364) using the following primers 5'-

349   CACACCACGTCTCATTAACCCTAGAAAGATAATCATATTGTG-3' and 5'-

350   CACACCACGTCTCACCCTAGAAAGATAGTCTGCGT-3' (the primers included BsmBI restriction

351   enzyme sites and overhangs corresponding to the left and right homology arms); 3-4) The left

352   and right homology arms, which consisted of 1kb genomic DNA fragments upstream and

353   downstream of the sgRNA sites respectively. Single base pair mutations were introduced into

354   the PAM sequence at each sgRNA binding site on the homology arms to prevent Cas9-

355   dependent cutting of the donor plasmid. The right homology arm also included the GFP coding

356   sequence in frame with the 3' end of the last coding exon of *sei*, immediately upstream of the

357   endogenous stop codon (Fig 6A). pDCC6 plasmids containing sgRNA and cas9 sequences (100

358   ng/μL) and the donor plasmid (500 ng/μL) were co-injected into y^-w^- background. Subsequently,

359   correct genomic integration of the GFP tag was verified by screening for DsRed-positive

360   animals, followed by sequencing of genomic PCR fragments. The final tagged *sei* allele was

361   generated by removing the DsRed cassette via the introduction of the *piggyBac* transposase

362   (Bloomington #8285) (Fig 6A).

363   The *sei-LexA* transgenic flies were generated by amplifying a 2612 bp genomic DNA fragment

364   upstream of the *sei* start codon by using the following PCR primers: 5'-

365   GTCGACCGCCGGCAAAGTATCAACAT-3' and 5'-GCGGCCGCTTTTAAGTCTGCAAAGTATAGAAACG-

366   3', followed by cloning into the pENTR1A plasmid (ThermoFisher) with SalI and NotI restriction

367   enzymes. The *sei* promoter fragment was then recombined into the pBPnlsLexA::p65Uw vector

368   (a gift from Gerald Rubin, Addgene plasmid # 26230)[52] by using the Gateway reaction

369   (ThermoFisher). The *sei-LexA* containing plasmid was integrated into the fly genome by using a

370   line carrying a PhiC31 integrase landing site on Chromosome III (Bloomington #24483)[94].

371   **Behavioral response to acute heat stress**

372   Assays were performed as previously described [25]. In short, two-day old flies were collected

373   and transferred into standard vials containing food (five per sex in each vial). On the following

374   day, flies were flipped into an empty vial, and tested within the next hour. For testing, vials with

375   10 flies were individually submerged into a 41-42°C water bath and observed for seizure-like

376   behavior and paralysis. The cumulative number of paralyzed flies (immobile at the bottom of

377   the vial) was recorded every 15 seconds. The time at which 50% of the flies in a given vial were

378   paralyzed was used as a measure of seizure susceptibility for statistical analysis.

379   **Behavioral response to gradual heat stress**

380   Two-day old flies were housed in groups of 10 as above. Subsequently, vials were placed in a

381   temperature-controlled incubator (Fisher Scientific Isotemp) with a glass door, which allowed

382   continuous video recording of their behavior. To test for the ability of flies to adapt to gradual

383   temperature increase, flies were first acclimated to 26°C followed by a 2°C increase every 30

384   minutes to a maximum of 42°C. The number of paralyzed flies was recorded every two minutes.

385   **Larval Locomotion Behavior**

386   A 60mm plastic petri dish was filled with 3% agar, and placed on a Peltier plate surface of a PCR

387   machine, which was set to either 37°C or 13°C for the heat or cold stress respectively. Identical

388   tests at 25°C were used as controls. For the heat stress condition, locomotion was assayed by

389   placing individual third instar foraging larvae on the agar surface and video recording (Logitech

390   C920 Webcam) for one minute. Larva locomotion was analyzed by assessing the amount of

391   time spent executing the following specific behaviors: peristaltic locomotion, whipping, head

392   thrashing, rolling and no movement [95](S1 Video). For the cold stress condition, individual

393   larvae were allowed to acclimate for 30s before their behavior was recorded for four min.

394   Behaviors of all larvae were tracked and analyzed by using a custom designed motion tracker

395   system (McKinney and Ben-Shahar, unpublished), which enabled computer-assisted analyses of

396   distance traveled.

397   **Larval nociceptive response to heat**

398   Foraging 3rd instar larvae were removed from bottles, washed in water, and placed on a water-

399   saturated 3% agar plate. Behavioral tests were conducted in constant 25°C and 70% humidity.

400   Larvae were allowed to acclimate to the agar plate for 10 seconds. A custom-made heat probe

401   (Thermal Solutions Controls and Indicators Corporation) set to 50°C was used to gently touch

402   the side of the larva's body, and the amount of time for the larva to roll over was recorded. For

403   each genotype, 47-50 larvae were individually tested.

404   **Immunostaining and imaging**

405   Third-instar larval brains were dissected and fixed in 4% PFA for 20 minutes, washed with

406   PBST+0.1% Triton-X (PBST), and then blocked with Superblock (ThermoFisher) for one hour.

407   Larval brains were subsequently incubated overnight at 4°C with rabbit anti-GFP (A-11122,

408   ThermoFisher) diluted in Superblock at 1:1000. After washing with PBST, larval brains were

409   incubated for 2 hours at room temperature with secondary antibody Alexa Fluor 488-

410   conjugated goat anti-rabbit (A-11034, ThermoFisher) and FITC-conjugated goat anti-HRP (123-

411   095-021, Jackson ImmunoResearch) to label neurons, each diluted 1:1000 in Superblock. After

412   secondary incubation, brains were washed in PBST, mounted using FluoroGel II with DAPI

413   (ThermoFisher), and imaged using an A1Si laser scanning confocal microscope (Nikon).

414   Adult brains were dissected, fixed and blocked as above, then subsequently incubated

415   overnight at 4°C in rabbit anti-GFP (A-11122, ThermoFisher) diluted at 1:1000 and mouse anti-

416   *Brp* (NC82; Developmental Studies Hybridoma Bank) diluted 1:33 in Superblock. After washing

417   with PBST, adult brains were incubated overnight at 4°C with secondary antibodies Alexa Fluor

418   488-conjugated anti-rabbit (A-11034, ThermoFisher) and Alexa Fluor rhodamine-conjugated

419   donkey anti-mouse (sc-2300, Santa Cruz Biotechnology), each diluted 1:500 in Superblock.

420   Adult brains were mounted and imaged as above. To image live brains expressing either

421   *RedStinger* or *GFPnls* nuclear markers, the tissues were dissected, mounted in PBS, and imaged

422   on a confocal microscope within one hour of the dissection.

423   **Statistical analysis**

424   Indicated statistical comparisons were analyzed by using Excel (Microsoft) and Prism 7

425   (GraphPad). Statistical significance was set at $p<0.05$.

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732

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737 studies.

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741   **Figure Captions**

742
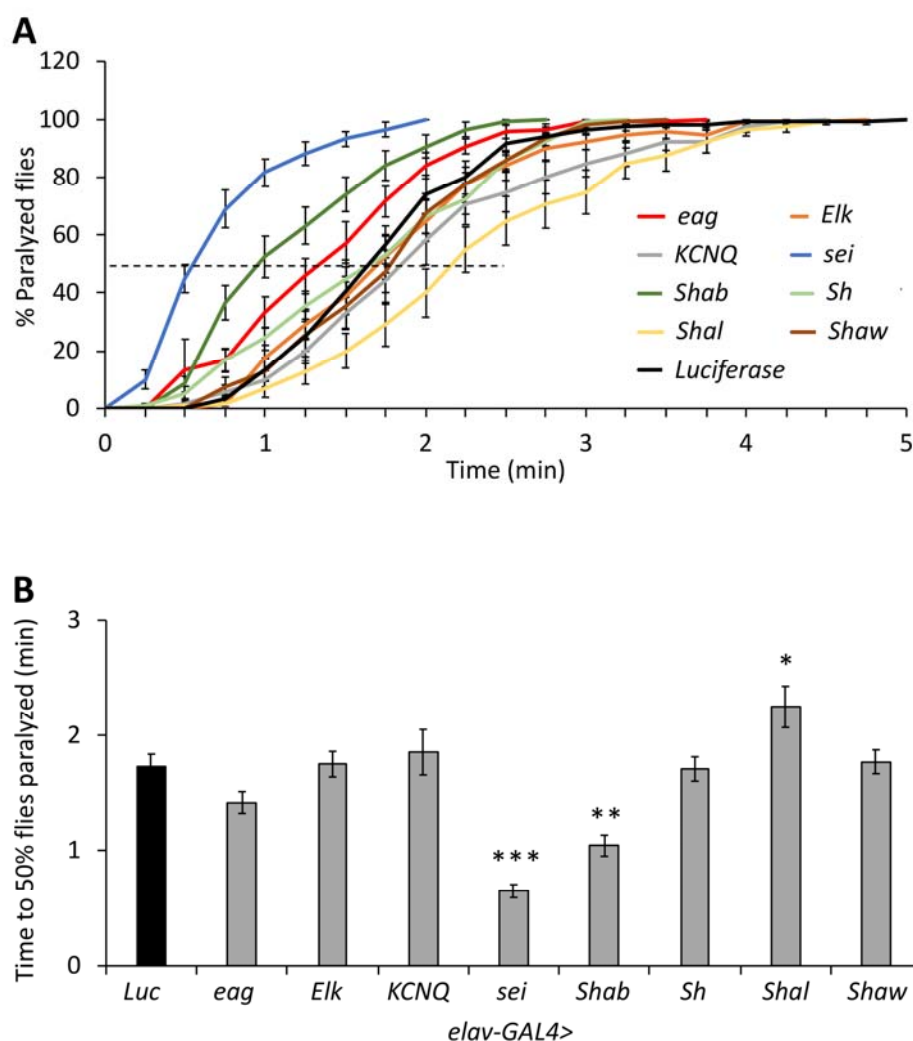
743   **Fig 1. Neuronal (*elav-GAL4*) RNAi knockdown screen of *Drosophila* genes encoding voltage**

744   **gated potassium channels.**

745   **A**) Cumulative percent paralyzed flies over time, with dotted line indicating 50% level. **B**) Direct

746   comparison of time at which 50% of flies are paralyzed, using the same data as in A. n=12

747   vials/genotype. ANOVA ($p < 0.0001$) followed by Dunnett's *post hoc* test was used to determine

748   groups significantly different than the *Luciferase* control. Data are presented as mean ±SEM, *p

749   < 0.05, **$p < 0.01$, ***$p < 0.001$.

Fig 2. *sei* mutants display heat hypersensitivity.

A-B) Acute heat assay paralysis behavior of homozygous and heterozygous *seiP* mutants and

controls. n=4 vials/genotype. Letters above bars represent significantly different groups by

ANOVA ($p < 0.001$) following Tukey's *post hoc* analysis ($p < 0.01$). **C-D**) Gradual heat assay

paralysis behavior of *seiP* mutant flies and controls. n=12 vials. **E**) Larval behavioral responses to

heat in *seiP* mutants and controls. n=9. **F**) Response time of *seiP* mutant larvae and controls to

thermal nociception assay. **G-H**) *seiP* mutant and wildtype larvae distance travelled in five

minute trials at room temperature (25°; n=6) or cold temperature (13°; n=12). Data are

presented as mean ±SEM, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (Student's *t*-test unless otherwise

stated).

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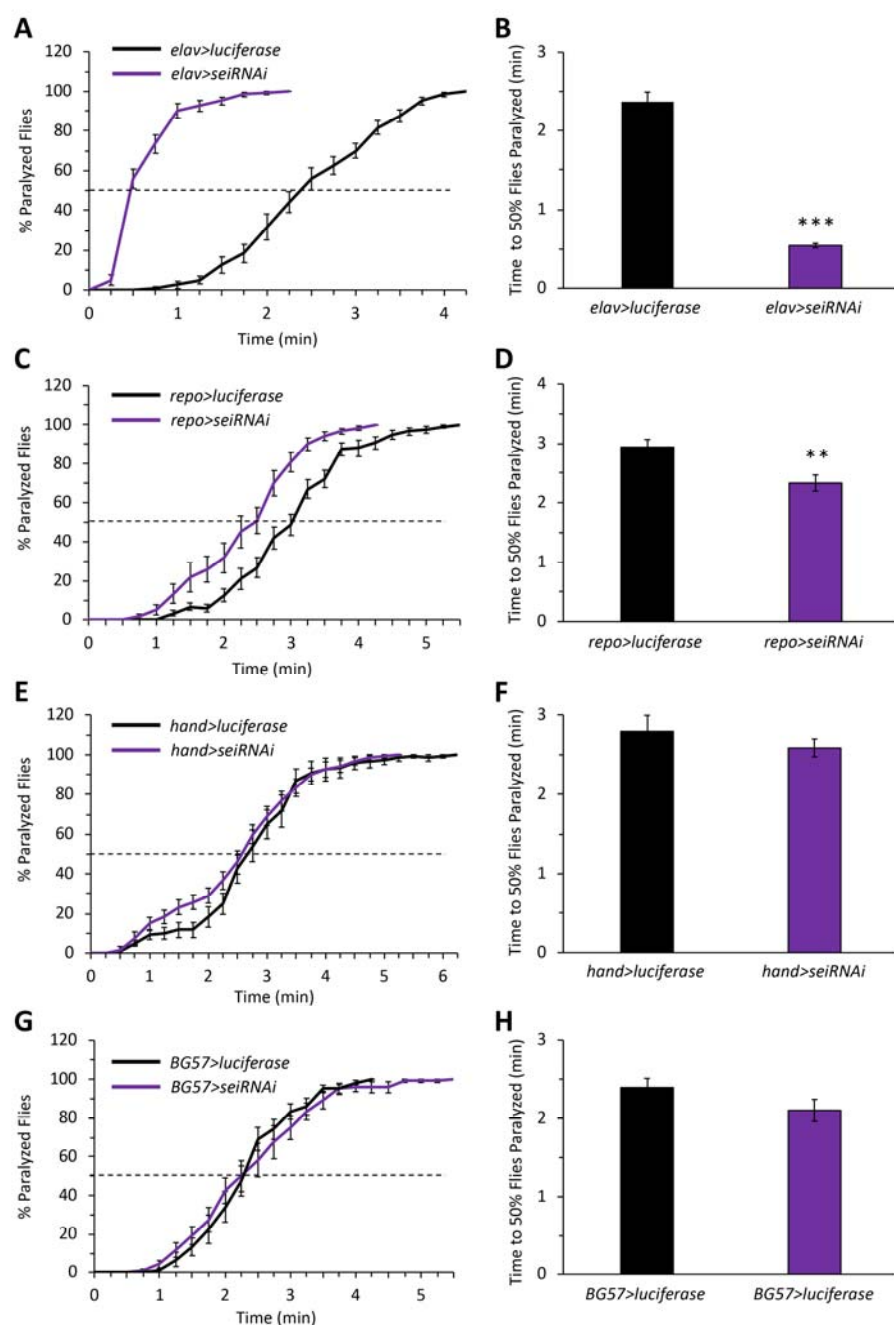
Fig 3. *sei* expression is required in neurons and glia for the homeostatic response to acute

heat stress.

A cell-type specific RNAi-dependent *sei* knockdown screen. **A-B**) Neurons (*elav-GAL4*); **C-D**) glia

(*repo-GAL4*); **E-F**) heart (*hand-GAL4*); **G-H**) muscle (*BG57-GAL4*). n=12 vials/genotype. Data are

presented as mean ±SEM, **$p < 0.01$, ***$p < 0.001$ (Student's *t*-test).

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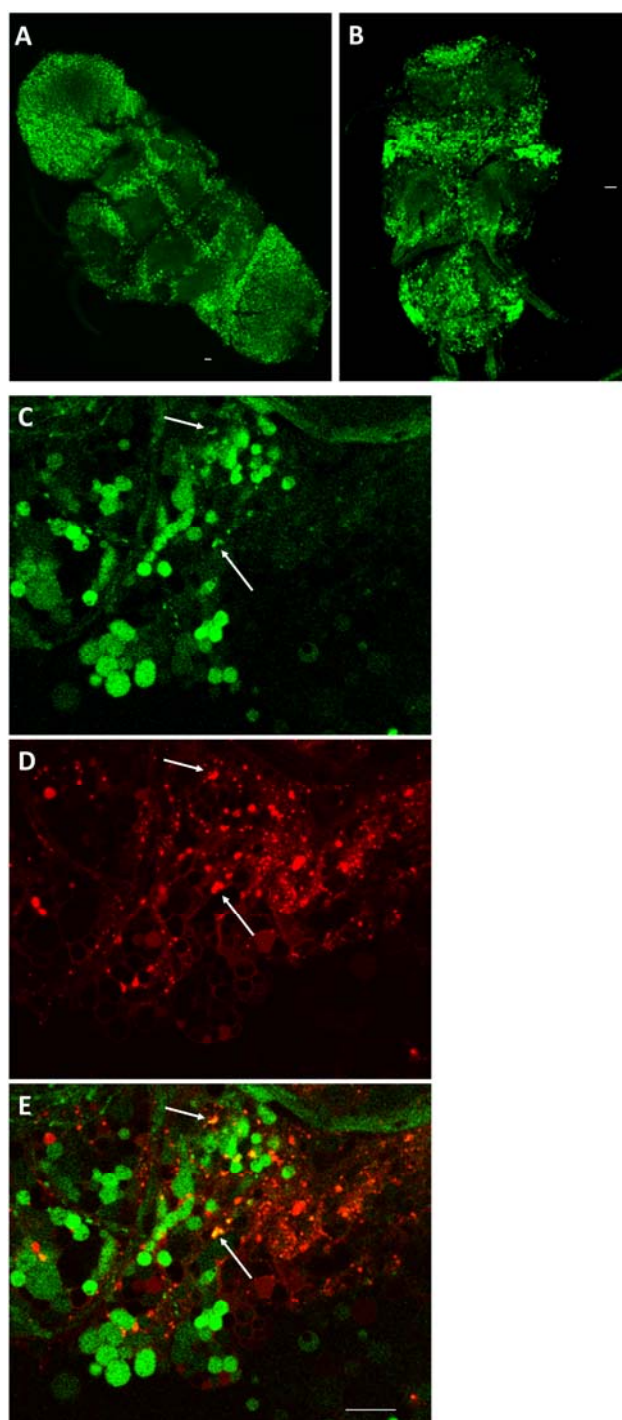
770 **Fig 4. *sei* promoter drives expression in neurons and glia.**

771 **A-B**) Representative confocal z-stack images of an adult brain (**A**) and ventral nerve cord (**B**)

772 from *sei-LexA>LexAOp-GFPnls* flies. **C-E**) Overlap of *sei-LexA>LexAOp-GFPnls* and glial marker

773 *RepoGAL4>UAS-RedStinger*. Arrows point to examples of co-labeled cells.
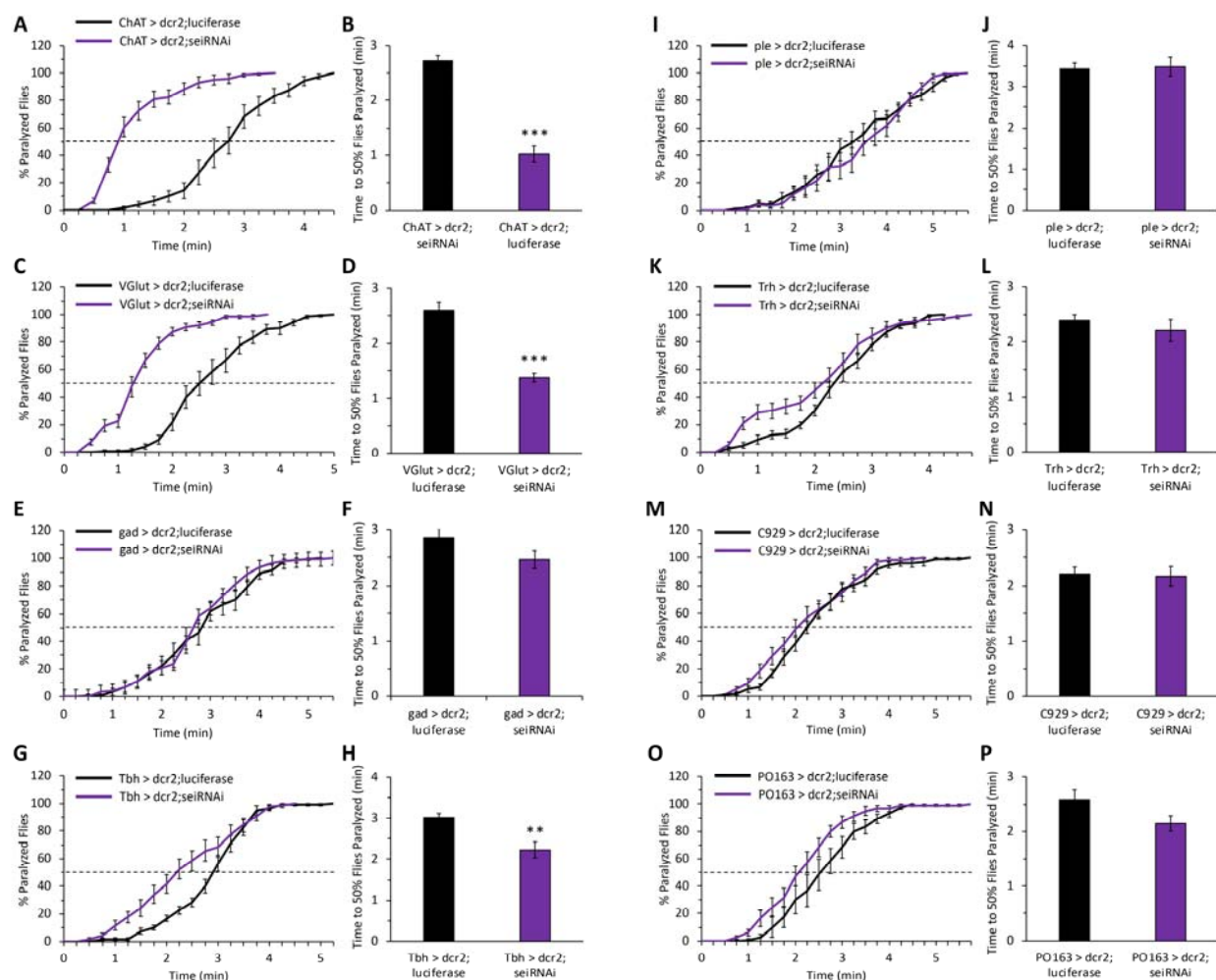
Fig 5. *sei* expression is specifically required in cholinergic, glutamatergic and octopaminergic cells for response to acute heat stress.

A neuronal subtype specific RNAi-dependent *sei* knockdown screen. **A-B**) Cholinergic neurons (*ChAT-GAL4*) n=12 vials/genotype; **C-D**) Glutamatergic neurons (*gad-GAL4*) n=6; **G-H**) Octopaminergic neurons (*Tbh-GAL4*) n=12; **E-F**) GABAergic neurons (*Gad1-GAL4*) n=12; **I-J**) Dopaminergic neurons (*ple-GAL4*) n=12; **K-L**) Serotonergic neurons (*Trh-GAL4*) n=12; **M-N**) Peptidergic neurons (*C929-GAL4*) n=6; **O-P**) Sensory neurons (*PO163-GAL4*) n=6. Data are presented as mean ±SEM, **$p < 0.01$, ***$p < 0.001$ (Student's *t*-test).

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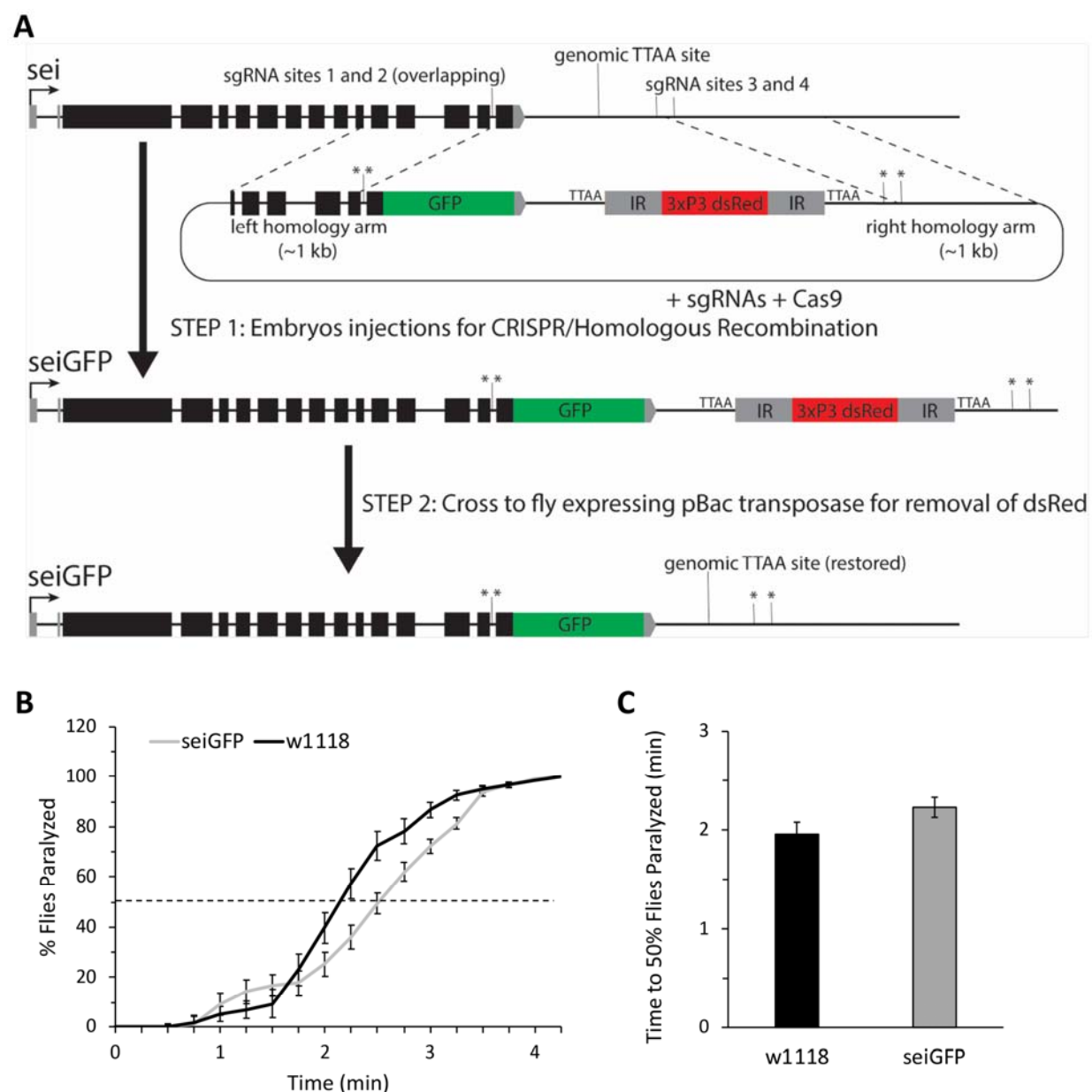
Fig 6. Generation and validation of the *seiGFP* allele.

A) Strategy for the generation of the *seiGFP* allele by using CRISPR/*Cas9*-dependent DNA

editing. Stars represent single nucleotide substitutions in the PAMs of sgRNA sites. **B-C**)

Behavior of *seiGFP* and wildtype flies in the acute heat assay. n=12. Data was analyzed using

Student's *t*-test and presented as mean ±SEM.

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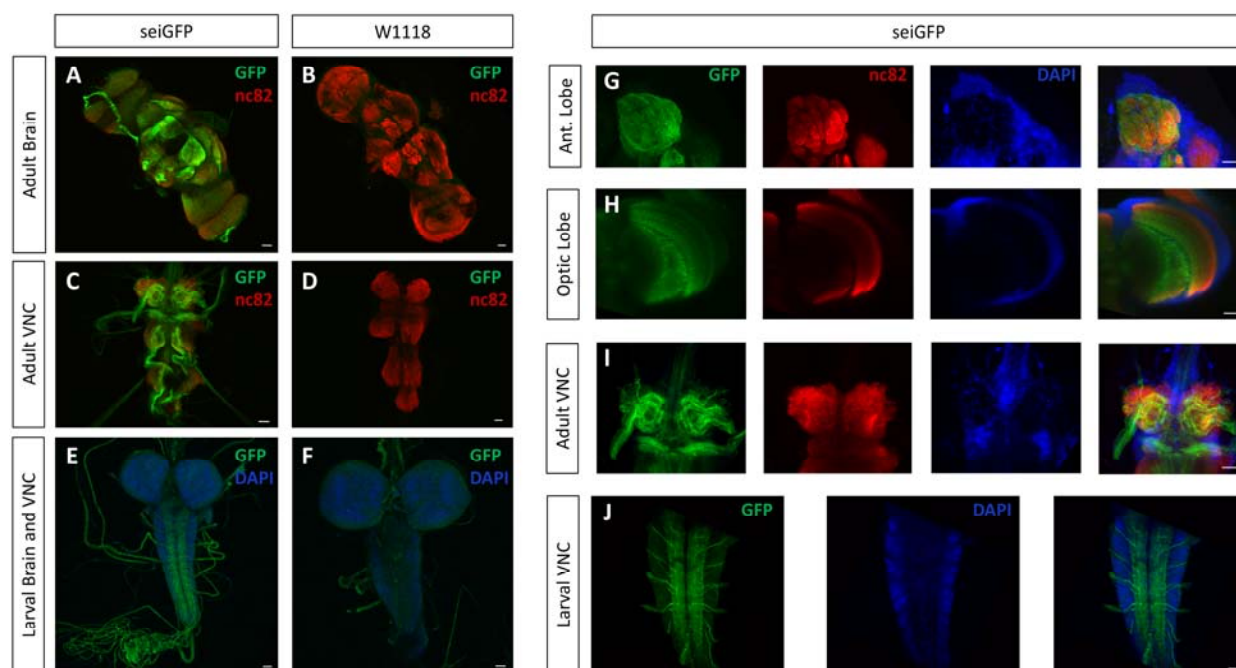
Fig 7. Nervous system expression of *seiGFP* in adult and larva is primarily localized to

neuronal processes.

A-D) Representative 20x confocal z-stack images of the adult brain (**A-B**) and adult ventral nerve

cord (**C-D**) following immunostaining for *seiGFP* (green) and nc82 (red). **E-F**) 20x confocal z-stack

images of the larval brain and ventral nerve cord of *seiGFP* (green) and DAPI (blue). **G-I**) 40x

confocal z-stack images of the adult antennal lobe (**G**), optic lobe (**H**) and ventral nerve cord (**I**)

with *seiGFP* (green), nc82 (red) and DAPI (blue). **J**) 40x confocal z-stack images of the larval

ventral nerve cord of *seiGFP* (green) and DAPI (blue). Scalebars are 20μm.

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Supporting Information

S1 Video. Heat induced *seiP* mutant larval behavior.