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KEK-6: A TRUNCATED-TRK-LIKE RECEPTOR FOR *DROSOPHILA* NEUROTROPHIN 2

REGULATES STRUCTURAL SYNAPTIC PLASTICITY

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28   ABSTRACT

29   Neurotrophism, structural plasticity, learning and long-term memory in mammals critically

30   depend on neurotrophins binding Trk receptors to activate tyrosine kinase (TyrK) signalling,

31   but *Drosophila* lacks full-length Trks, raising the question of how these processes occur in

32   the fly. Paradoxically, truncated Trk isoforms lacking the TyrK predominate in the adult

33   human brain, but whether they have neuronal functions independently of full-length Trks is

34   unknown. *Drosophila* has TyrK-less Trk-family receptors, encoded by the *kekkon (kek)*

35   genes, suggesting that evolutionarily conserved functions for this receptor class may exist.

36   Here, we asked whether Keks function together with Drosophila neurotrophins (DNTs) at the

37   larval glutamatergic neuromuscular junction (NMJ). Starting with an unbiased approach, we

38   tested the evelen LRR and Ig-containing (LIG) proteins encoded in the *Drosophila* genome

39   for expression in the central nervous system (CNS) and potential interaction with DNTs. Kek-

40   6 was expressed in the CNS, could interact genetically with DNTs and could bind DNT2 both

41   in signaling essays and in co-immunoprecipitations. There is promiscuity in ligand binding, as

42   Kek-6 could also bind DNT1, and Kek-5 could also bind DNT2. In vivo, Kek-6 is found

43   presynaptically in motoneurons, and binds DNT2 produced by the muscle, which functions as

44   a retrograde factor at the NMJ. Kek-6 and DNT2 regulate NMJ growth, bouton formation and

45   active zone homeostasis. Kek-6 does not antagonise the alternative DNT2 receptor Toll-6,

46   but rather the two receptors contribute in distinct manners to NMJ structural plasticity. Using

47   pull-down assays, we identified and validated CaMKII and VAP33A as intracellular partners

48   of Kek-6, and show that together they regulate NMJ growth and active zone formation. These

49   functions of Kek-6 could be evolutionarily conserved, raising the intriguing possibility that a

50   novel mechanism of structural synaptic plasticity involving truncated Trk-family receptors

51   independently of TyrK signaling may also operate in the human brain.

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56   AUTHOR SUMMARY

57   A long-standing paradox had been to explain how brain structural plasticity, learning and

58   long-term memory might occur in Drosophila in the absence of canonical Trk receptors for

59   neurotrophin (NT) ligands. NTs link structure and function in the brain enabling adjustments

60   in cell number, dendritic, axonal and synaptic patterns, in response to neuronal activity.

61   These events are essential for brain development, learning and long-term memory, and are

62   thought to depend on the tyrosine-kinase function of the NT Trk receptors. However,

63   paradoxically, the most abundant Trk isoforms in the adult human brain lack the tyrosine

64   kinase, and their neuronal function is unknown. Remarkably, Drosophila has kinase-less

65   receptors of the Trk family encoded by the *kekkon (kek)* genes, suggesting that deep

66   evolutionary functional conservation for this receptor class could be unveiled. Here, we show

67   that Kek-6 is a receptor for Drosophila neurotrophin 2 (DNT2) that regulates structural

68   synaptic plasticity via CaMKII and VAP33A, well-known factors regulating synaptic structure

69   and plasticity, and vesicle release. Our findings suggest that in mammals truncated Trk-

70   family receptors could also have synaptic functions in neurons independently of Tyrosine

71   kinase signalling. This might reveal a novel mechanism of brain plasticity, with important

72   implications for understanding also the human brain, in health and disease.

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84   INTRODUCTION

85   Brain plasticity, neurotrophism in development, structural and synaptic plasticity during

86   learning and long-term memory in humans critically depend on the receptor TrkB binding its

87   neurotrophin (NT) ligand BDNF [1,2]. During development, NTs and Trks regulate neuronal

88   number and connectivity; subsequently BDNF and TrkB establish a reinforcing positive

89   feedback loop promoting synaptic potentiation, and regulate the dynamic generation and

90   elimination of synaptic boutons and dendritic spines in response to activity [1,3-5]. Thus, NTs

91   and Trks are fundamental to linking structure and function in the brain, and this is thought to

92   depend mostly on the tyrosine kinase (TyrK) function of Trks. Through its intracellular TyrK

93   domain, TrkB activates the Ras/MAPKinase, PI3Kinase/ AKT and PLCγ signalling pathways

94   downstream [1,2]. Pre-synaptic targets include Synapsin, to trigger vesicle release [5]. Post-

95   synaptic targets include NMDAR and CREB, essential for long-term potentiation, learning

96   and long-term memory [1,5]. Paradoxically, full-length TrkB decays postnatally and TrkB

97   homodimers are not formed in the adult mammalian brain [6-10]. Instead, the most abundant

98   adult isoform is the truncated TrkB-T1 isoform lacking the TyrK [8-10]. Mutant mice lacking

99   TrkB-T1 have anxiety, and in humans alterations in TrkB-T1 are linked to severe mental

100   health disorders [11-13]. However, the neuronal functions of the truncated Trk isoforms are

101   poorly understood.

102   No canonical, bona fide, full-length, TyrK Trk receptors have been found in

103   *Drosophila*. However, neurotrophism, structural and synaptic plasticity, learning and long-

104   term memory all occur in fruit-flies, implying that either in the course of evolution insects and

105   humans found different molecular solutions to elicit equivalent functions, or that undiscovered

106   mechanisms contribute to brain plasticity in both humans and fruit-flies. Finding out what

107   happened to the Trks in *Drosophila* is important, as it could uncover novel fundamental

108   mechanisms of structure-function relationships in any brain.

109   Trk receptors have long been searched for in *Drosophila*. Trks (TrkA,B,C) bear the

110   unique combination of Cysteine Rich Repeats (CRR), Leucine Rich Repeats (LRR) and

111   Immunoglobulin (Ig) domains extracellularly, and an intracellular TyrK domain (Fig.1A).

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112   Original searches focused on the TyrK domain, and identified DTrk and Dror as candidate

113   *Drosophila* Trk homologues, but these are unlikely to bind neurotrophins. DTrk, also known

114   as Off-Track (Otk)[14,15] lacks the LRR and CRR modules, it is kinase-dead and binds

115   Semaphorins (Fig.1A). Dror/Dnrk, like all Ror-family receptors, has an extracellular

116   Frizzled/Kringle module instead [16-18](Fig.1A). Subsequent proteomic analyses found no

117   full-length, canonical Trk orthologues with the combination of LRR, CRR and Ig modules

118   extracellularly and a Trk-family TyrK intracellularly in *Drosophila* [19-21]. However,

119   phylogenetic analysis of the Trk-receptor superfamily identified the *Drosophila* Kekkons

120   (Keks), lacking an intracellular TyrK domain, as closely related to the Trks [22](Fig.1A,B).

121   Trks and Keks both belong to the LIG family of proteins that contain extracellular ligand-

122   binding LRR and Ig motifs [22,23]. There are 38 LIGs in humans, and amongst these are

123   transmembrane proteins with a divergent intracellular domain lacking a TyrK or any

124   conserved motifs [22]. There are 9 LIGs in *Drosophila* and phylogenetic analysis clusters

125   mammalian AMIGO, LINGO, LRIG and LRRC4 in one clade with *Drosophila* Lambik, and

126   mammalian Trks in a separate clade together with *Drosophila* Keks (Kek1-6) [22](Fig.1B).

127   Keks are more similar to the Trks than all other vertebrate LIGs are to each other [22]. Keks

128   have only been found in insects, and thus are remnant, conserved Trk-like receptors in fruit-

129   flies.

130   There are 6 Keks in *Drosophila*, with a cytoplasmic tail lacking any remarkable

131   conservation, except that Kek1, 2, 5 and 6 have a PDZ domain that could bind cytoplasmic

132   effectors [24]. Kek1, 2, 5 and 6 are highly conserved in insects [24]. At least *kek1, kek2* and

133   *kek5* are expressed in the CNS [25-27]. Only Kek2 has been investigated in the CNS, where

134   it functions as a neuronal activity dependent modulator of synaptic growth and activity [27].

135   The ligands for the Keks have not been identified.

136   The prime candidates for Kek ligands are the *Drosophila* neurotrophins (DNTs).

137   DNT1 (*Drosophila* neurotrophin 1 also known as Spz2), DNT2 (also known as Spz5) and Spz

138   bear the distinctive and evolutionarily conserved neurotrophin cystine-knot domain of the

139   mammalian neurotrophins [28-36], and they have conserved CNS functions regulating

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140   neuronal survival, connectivity and synaptogenesis [35-39]. Spz, DNT2 and DNT1 are known

141   ligands for Toll-1, Toll-6 and Toll-7 receptors of the Toll and Toll-Like Receptor (TLR)

142   superfamily [38,40]. In *Drosophila* Toll-1, Toll-6 and Toll-7 are required for targeting at the

143   embryonic neuromuscular junction (NMJ), Toll-6 and Toll-8 for larval NMJ growth, and Toll-1,

144   Toll-6 and Toll-7 function as neurotrophin receptors regulating neuronal survival and death,

145   connectivity and behaviour [35-38,41-44]. In *Drosophila*, the NMJ is glutamatergic, and

146   undergoes plasticity and potentiation, thus resembling mammalian central synapses, and is

147   the standard context in which to investigate synaptic structural and functional plasticity [45].

148   Given that NT family ligands can bind multiple receptor types, and that receptors and ligands

149   tend to co-evolve [17], conservation of the extracellular ligand-binding domain of Trks and

150   Keks suggested Keks could potentially function as DNT receptors in flies.

151   Here, we investigated whether Kek-6 might function as a DNT receptor in *Drosophila,*

152   at the glutamatergic NMJ synapse.

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154   RESULTS

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156   **Kek6 is a truncated-Trk-like receptor that binds DNTs**

157   To investigate if *Drosophila* LIGs might function as DNT receptors in the CNS, we first looked

158   at their expression in embryos. *CG15744* did not reveal expression above background in the

159   ventral nerve cord (VNC) (Fig.1C); *lambik* and *CG16974* mRNAs were absent from the VNC,

160   but might be expressed in the Peripheral Nervous System (PNS) or muscle, respectively

161   (Fig.1C,D). *kek1* and *kek2* were expressed in VNC cells, and possibly in PNS cells too

162   (Fig.1D), as previously shown [25,27]. *kek3* and *kek4* transcripts were not detected in

163   embryos. *kek5* is expressed in the VNC [26], and we confirmed this with a *kek5-GAL4*

164   reporter, which also revealed PNS expression (Fig.1D). *kek6* transcripts were abundant in

165   the VNC (Fig.1D). Thus, amongst the 9 LIGs, Kek1, 2, 5, and 6 could function in the CNS.

166   To test whether Keks could function downstream of DNTs in vivo, we took advantage

167   of the cold semi-lethality of *DNT1^{41} DNT2^{e03444}* double mutants [38], and asked whether it

168   could be rescued with the over-expression of *keks* in neurons. Over-expression of *kek1* and

169   *kek4* in neurons (with *elavGAL4)* did not rescue, and *kek2* and *kek6* did most prominently

170   (Fig.1E, Table S1). Thus, Kek2 and Kek6 could function downstream of DNTs.

171   To ask whether DNT ligands could bind Keks and induce signaling, as Keks lack the

172   TyrK, to enable a signaling readout, we generated chimaeric receptors formed of the

173   extracellular and transmembrane domains of the Keks fused to the intracellular domain of

174   Toll-6 (Fig.2A). Toll-6 uses a conserved TIR domain to activate Dif/NFκB signaling

175   downstream, which can be measured through the activation of *drosomicin-luciferase (dros-*

176   *luc)* [38]. We used S2 cells stably transfected with *dros-luc*, transfected them with *kek-Toll-6*

177   chimaeric receptors, and tested whether stimulation with purified cleaved DNT2 (DNT2-CK)

178   could induce Dif/NFκB signaling. The chimaeric receptors targeted correctly to the S2 cell

179   membrane (Fig.2A). Stimulation with DNT2-CK in pDONR transfected controls induced *dros-*

180   *luc,* as S2 cells express multiple Toll receptors [36,38] (Fig.2A). Stimulation with DNT2-CK of

181   cells transfected with *kek3,4,5,6-Toll-6* chimaeras had an effect comparable to the induction

182   by stimulated full-length *Toll-6,* and Kek3-Toll-6 and Kek6-Toll-6 chimaeras responded more

183   robustly (Fig.2A, Table S1). We could not generate Kek1 and Kek2 chimeric receptors, thus

184   a potential interaction with them cannot be ruled out. Thus, DNT2 can interact physically with

185   at least Kek-3 and Kek-6. Together with the above data, this indicated Kek-6 was a strong

186   candidate to interact with DNTs in the CNS.

187   To verify whether Kek-6 could bind DNT2, we carried out co-immunoprecipitations.

188   S2 cells were co-transfected with HA-tagged *kek-6* and V5-tagged full-length *DNT2.*

189   Precipitating Kek6-HA with anti-HA, brought down DNT2-V5 detected with anti-V5 (Fig. 2B).

190   Conversely, precipitating DNT2 with anti-V5, also brought down Kek6 detected with anti-HA

191   (Fig. 2C). Thus, Kek6 can bind DNT2. To test whether Kek6 might also bind DNT1, we co-

192   transfected S2 cells with *kek6-HA* and full-length *DNT1-V5.* Precipitating DNT1 brought down

193   Kek6 (Fig. 2C). Thus, Kek6 can bind both DNT2 and DNT1. To test whether another Kek

194   might also bind the DNTs, S2 cells were co-transfected with *kek5-Flag* and *DNT2-V5*, and

195   we found that precipitating DNT2 also brought down Kek-5 (Fig. 2D). Therefore, Kek-5 can

196   also bind DNT2. These data demonstrate that Keks bind DNT ligands, and that binding is

197   promiscuous.

198   To conclude, Kek-6 was widely expressed in the CNS, rescued the semi-lethality of

199   *DNT1 DNT2* double mutants, and bound DNT ligands in a signaling assay and in co-

200   immunoprecipitations. Thus, Kek-6 could function as a DNT receptor.

201

202   **Kek-6 in motoneurons and DNT2 from the muscle function together at the NMJ**

203   For a functional *in vivo* analysis we focused on Kek-6. *kek-6* CNS expression was examined

204   in larvae using Kek6^{MIMIC13953} flies bearing a GFP insertion into the *kek-6* coding region

205   (hereafter named Kek6^{GFP}). Kek6^{GFP} was found in Eve+(Fig.3A) and HB9+(Fig.3B) inter-

206   neurons and motoneurons, and excluded from Repo+ glia (Supplemental Fig.S1) in third

207   instar larval VNCs. Kek6^{GFP} was present in motoneuron terminals at the neuro muscular

208   junction (NMJ) of third instar larvae and not in the muscle (Fig.3C,D), revealing NMJ6/7

209   synaptic boutons, surrounded by post-synaptic Dlg (i.e. *Drosophila* PSD95) (Fig.3D,E). Thus,

210   *kek-6* is expressed pre-synaptically in motoneurons.

211   *DNT2* transcripts are expressed in the larval body wall muscles, and localised to post-

212   synaptic boutons [39]. To test if DNT2 could function retrogradely, we generated a tagged

213   form of full-length DNT2 with GFP at the C-terminus, and over-expressed *DNT2-FL-GFP* in

214   the muscle with *MhcGAL4.* DNT2-FL-GFP would result in secretion of mature DNT2-CK-GFP

215   [36], although not all protein might get cleaved and secreted. Over-expression of *DNT2-FL-*

216   *GFP* in muscle resulted in the localization of GFP pre-synaptically in boutons, surrounded by

217   the post-synaptic marker Dlg (Fig.3F). DNT2-FL-GFP also colocalised with the motoneuron

218   marker FasII in boutons (Fig.3G,H,J) and in motoraxons (Fig.3H,I,J). Thus, DNT2 produced

219   in muscle can interact with the receptor Kek-6 in motoneurons, consistent with a retrograde

220   function.

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221   To investigate the *in vivo* functions of Kek6 and DNT2, we generated *kek6* and *DNT2*

222   null mutant alleles by FRT-mediated recombination between PiggyBac insertions [46].

223   Neither *kek6^{34}/Df(3R)ED6361* or *DNT2^{37}/Df(3L)6092* loss of function mutants, nor

224   *kek6$^{-/-}$ DNT2$^{-/-}$* double mutants, affected viability. We analysed larval locomotion using

225   FlyTracker software to trace trajectories and measure crawling speed [38], and found that

226   *kek6^{34}/Df(3R)ED6361* mutant larvae crawled more slowly than controls (Fig.4A, Table S1),

227   and *DNT2^{37}/Df(3L)6092* larvae crawled even slower (Fig.4A, Table S1). *kek6$^{-/-}$ DNT2$^{-/-}$*

228   double null larvae crawled at similar speeds as *DNT2$^{-/-}$* mutants, but travelled shorter

229   distances, moving around the starting spot (Fig.4A, Table S1). The synergistic effect in the

230   double mutants suggested that Kek-6 and DNT2 are functionally linked.

231   Locomotion phenotypes suggested the NMJ might be affected, so we looked at the

232   muscle 6/7 NMJs, which require DNT1 and 2 [39]. Targeting at muscle 6/7 NMJ was affected

233   in *kek6$^{-/-}$* mutant embryos and upon *kek-6* over-expression in neurons (Supplemental

234   Fig.S2). In wandering third instar larvae, *kek6$^{-/-}$* and *DNT2$^{-/-}$* single mutant larvae, and *kek6^{-}*

235   *$^{/-}$ DNT2$^{-/-}$* double mutants, had smaller NMJs than controls, with fewer Ib boutons and

236   shorter axonal terminal length (Fig.4B, Table S1). Thus, Kek-6 and DNT2 are required for

237   normal NMJ growth. *kek6$^{-/-}$* and *DNT2$^{-/-}$* single mutant NMJs had higher active zone density,

238   visualized with anti-Brp (*Drosophila* ELKs) and quantified automatically throughout the NMJ

239   stack of images using DeadEasy Synapse software [39](Fig.4B, Table S1). Since the NMJs

240   were smaller, this suggested that the increase in active zones was a homeostatic

241   compensation of defective synaptic function, to enable adequate behaviour. Homeostatic

242   adjustments in active zones are a common manifestation of structural plasticity at the NMJ

243   [47]. Remarkably, the increase in active zone density did not occur in *kek-6$^{-/-}$ DNT2$^{-/-}$* double

244   mutants (Fig.4B, Table S1), meaning that compensation fails in the double mutants. To

245   further test how Kek-6 affects the synapse, we also visualised Synapsin, which

246   phosphorylates components of the SNARE complex to promote neurotransmitter vesicle

247   release [48], and quantified it automatically using DeadEasy Synapse software [39]. *kek6$^{-/-}$*

248   mutants had reduced Synapsin production (Fig.4B, Table S1), revealing defective synapse

249   composition. Conversely, over-expression of *kek-6* did not alter NMJ size (Fig.5A and

250   Supplemental Fig.S4A), but it increased active zone density (Fig.5A, Table S1), and induced

251   ghost boutons, albeit not significantly (Supplemental Fig.S3C-E, Table S1). Ghost boutons

252   are presynaptic, HRP+ Dlg-negative, immature boutons that fail to get stabilized, and are

253   correlates of increased neuronal activity, thus further indicating that Kek-6 influences

254   structure and/or function. Like Kek-6 gain of function, over-expression of full-length *DNT2-FL*

255   in muscle, and either full-length *DNT2-FL* or mature *DNT2-CK* in motorneurons (with

256   *D42GAL4*), also increased active zones (Fig.5B,C Table S1). Thus both DNT2 and Kek6

257   induce active zone formation. Altogether, these data show that both Kek-6 and DNT2 are

258   required to regulate NMJ growth and for appropriate synaptic structure.

259   Our data suggested that DNT2 functions as a retrograde ligand for Kek-6. Over-

260   expression of *DNT2-FL* in muscle increased axonal terminal size and pre-synaptic active

261   zones (Fig.5B). To verify this, we used epistasis analysis. Over-expression of *kek6* in

262   motorneurons rescued the NMJ mutant phenotypes of bouton number and axonal length of

263   *kek6^{34}/Df(3R)6361* mutants (Fig.6A, Table S1)*,* demonstrating that the *kek6–/–* mutant

264   phenotypes were specific. Over-expression of *kek6* in motorneurons rescued the NMJ

265   phenotypes of *DNT2^{37}/Df(3L)6092* mutants and *kek6$^{-/-}$ DNT2$^{-/-}$* double mutants (Fig.6B,C,

266   Table S1), demonstrating that Kek-6 functions downstream of DNT2. Over-expression of

267   untagged *DNT2-FL* in muscle (with *MhcGAL4*) increased Dlg+ bouton number (Fig.6B), and

268   importantly, this was rescued by *kek-6* loss of function (Fig.6B, Table S1). Since Kek6

269   functions pre-synaptically, and DNT2 was over-expressed in muscle, this demonstrates that

270   DNT2 is a retrograde ligand for Kek-6. Altogether, our data demonstrate that DNT2 is a

271   retrograde ligand for Kek-6 at the NMJ and that DNT2 and Kek6 are required together for

272   synaptic structure and NMJ growth.

273   Intriguingly, our data also suggested that DNT2 had additional functions compared to

274   *kek-6*. Homeostatic compensation of active zones did not occur in *kek-6$^{-/-}$ DNT2$^{-/-}$* double

275   mutants (Fig.4B). Furthermore, over-expression of *DNT2* but not *kek-6* increased NMJ size,

276   as both HRP+ axonal terminal length (Fig.5B) and bouton number (Fig.6B) increased when

277   *DNT2-FL* was over-expressed from muscle. DNT2 is also a known ligand of Toll-6 [38], and

278   Toll-6 and -8 are required in motorneurons for NMJ growth and active zone formation[37,43].

279   So this raised two questions: do Toll-6 and Kek-6 interact functionally as DNT2 receptors at

280   the NMJ, and why?

281

282   **Kek-6 and Toll-6 cooperate to modulate NMJ structural homeostasis**

283   LIG proteins and truncated Trk isoforms can function as ligand sinks or dominant negative

284   co-receptors that abrogate signaling, e.g. by full-length Trks [6,7]. Thus, Kek-6 might inhibit

285   Toll-6 function.

286   Although Toll-6 has NMJ functions, its expression here had not been reported [43].

287   Using a MIMIC insertion into the intronless coding region of Toll-6, we generated *Toll-6GAL4*

288   flies by RMCE, and found Toll-6>mCD8-GFP to be distributed at the muscle 6/7 NMJ,

289   colocalising with the motoraxon marker FasII (Fig.7A). Like *kek-6* and *DNT2* mutants, *Toll-*

290   *6^{MIO2127}/Df(3L)BSC578* mutants had smaller NMJs, with shorter HRP+ axonal length (Fig.6B,

291   Table S1) as previously reported [43], confirming that Toll-6 is required for NMJ growth.

292   Contrary to *kek-6^{-/-}* mutants, *Toll-6^{-/-}* mutants had reduced Brp+ active zones compared to

293   controls (Fig.7B), meaning that Toll-6 is required for active zone formation. *kek-6^{-/-} Toll-6^{-/-}*

294   double mutant larvae still had small NMJs, like each single mutant alone (Fig.7B, Table S1),

295   and Brp+ active zones remained lower than in controls and comparable to the levels of *Toll-*

296   *6^{-/-}* single mutants (Fig.7B). These results reveal that: 1) Since Toll-6 is required for active

297   zone formation, and over-expression of *kek-6* increased active zones, Kek-6 does not

298   function as a DNT2 sink nor as an inhibitor or dominant negative co-receptor for Toll-6. 2)

299   The compensatory increase in active zones observed in *kek-6^{-/-}* and *DNT2^{-/-}* single mutants

300   did not occur in *kek-6^{-/-} DNT2^{-/-}* or *kek-6^{-/-} Toll-6^{-/-}* double mutants, meaning that the

301   concerted functions of Toll-6 and Kek-6 as DNT2 receptors are required for NMJ structural

302   homeostasis.

303   To test whether Kek-6 and Toll-6 might physically interact to form a receptor complex,

304   we carried out co-immunoprecipitations, and to ask if potential interactions were promiscuous

305   or specific, we also tested Toll-7. S2 cells were co-transfected with the active forms *Toll-6^{CY}-*

306   *Flag* or *Toll-7^{CY}-Flag* and *Kek6-HA*, and we found that precipitating Toll-6^{CY} and Toll-7^{CY} with

307   anti-Flag brought down Kek6 detected with anti-HA (Fig.7C). Thus, Kek6 can bind Toll-6 and

308   Toll-7.

309   Altogether, these data showed that retrograde DNT2 can bind two receptor types at

310   the NMJ - Toll-6 and Kek-6 - which can interact pre-synaptically to cooperatively promote

311   NMJ growth and regulate active zone homeostasis. This, however, raised a new question: if

312   Kek-6 did not function simply by modulating Toll-6, and in the absence of a TyrK, how might

313   it function?

314

315   **Kek-6 functions via CaMKII and VAP33A**

316   To find out how Kek-6 might function, we carried out pull-down assays to isolate candidate

317   factors binding its intracellular domain. S2 cells were transfected with *kek6-Flag,* and anti-

318   Flag coated beads were used to expose Kek-6 to cell lysates from either S2 cells or wild-type

319   adult fly heads, and bound proteins were isolated by SDS-PAGE followed by mass

320   spectrometry (Fig.8A,B). Candidates were identified as proteins present in Kek6-Flag

321   samples and absent from non-transfected mock controls, and if identified from multiple

322   peptides (Tables S2 and S3). Prevalent amongst these were proteins involved in vesicle

323   trafficking, axonogenesis, dendrite morphogenesis and synaptic function (Fig.8C). Amongst

324   the top hits were CaMKinase II identified from fly heads, and VAP33A from both S2 cells and

325   heads (Fig.8C). CaMKII functions both as a kinase and a scaffolding protein, to promote

326   structural synaptic plasticity. Post-synaptically, it phosphorylates and recruits AMPAR and

327   NMDAR to the post-synaptic density, leading to post-synaptic potentiation, and pre-

328   synaptically it localizes to active zones and phosphorylates Synapsin and other SNARE

329   complex proteins, triggering neurotransmitter release [48-51]. VAP33A is a Vamp Associated

330   Protein, of the SNARE complex, and also has universal functions in exocytosis and vesicle

331   trafficking [52]. To validate these two candidates as downstream effectors of Kek-6, we

332   carried out co-immunoprecipitations. We co-transfected S2 cells with Kek6-Flag and either

333   CaMKII or VAP33A tagged with HA. Precipitating Kek-6-Flag, brought down CaMKII-HA

334   (Fig.8D). Similarly, precipitating Kek-6-Flag also brought down VAP33A-HA (Fig.8D). Thus,

335   Kek-6 can bind CaMKII and VAP33A.

336   To validate the functional relationship between Kek-6 and CaMKII *in vivo*, we asked

337   whether altering Kek-6 function would affect CaMKII activation in brain. We tested for the

338   constitutively active state of CaMKII, corresponding to phosphorylation at Thr286 (T287 in

339   *Drosophila*), using anti-pCaMKII^{T287} antibodies. In the heads of *kek6^{34}/Df(3R)ED6361* mutant

340   adult flies the relative levels of pCaMKII^{T287}, normalised over non-phosphorylated CaMKII,

341   decreased (Fig.8E Table S1). Conversely, over-expression of *kek-6* in retina (with

342   *GMRGAL4*) increased CaMKII phosphorylation (Fig.8E, Table S1). Over-expressing *DNT2-*

343   *FL* had the same effect (Fig.8E). Thus, Kek-6 is required for CaMKII activation, and both

344   Kek-6 and DNT2 can activate CaMKII downstream.

345   Next we asked whether CaMKII and VAP33A might function downstream of Kek6 at

346   the NMJ. Unfortunately, we were not able to get antibodies to inactive CaMKII to work

347   reliably at the NMJ for normalisation, so we visualised constitutively activated CaMKII with

348   pCaMKII^{T287} and quantified it automatically, using DeadEasy Synapse, which detected the

349   increase in pCaMKII caused by the over-expression of activated CaMKII^{T287} in motoneurons

350   (*D42>CaMKII^{T287D}*, Fig. S4A,C). Pre-synaptic over-expression of *CaMKII^{T287}* increased

351   axonal length, Ib bouton number and active zone density, and inhibiting CaMKII activation

352   pre-synaptically by over-expressing the CaMKII phosphorylation inhibitor *Ala* [53] resulted in

353   smaller NMJs and reduced active zones (Fig.S4A-C, Table S1), as previously reported [54].

354   Thus we asked whether Kek-6 influenced CaMKII at the NMJ. Pre-synaptic over-expression

355   of *kek6* increased pCaMKII^{T287} levels (Fig.9A, Table S1). This increase was rescued by the

356   over-expression of *Ala* together with *kek6,* showing that this phenotype was specific (Fig.9A,

357   Table S1). However, over-expression of *Ala* alone did not result in a detectable reduction in

358   pCaMKII in this case (Fig.9A, Table S1). This could be due either to the fact that CaMKII is

359   most abundant post-synaptically, and we were only inhibiting it pre-synaptically, or to limited

360   efficacy of the inhibitor. To try an alternative approach, we knocked-down pre-synaptic

361   *CaMKII* expression with RNAi, and this decreased pCaMKII^{T287} levels, causing a stronger

362   effect than Ala (Fig.9A, Table S1). Like Ala, pre-synaptic *CaMKII-RNAi* knock-down together

363   with *kek-6* over-expression also rescued pCaMKII^{T287} levels, and reduced them further than

364   controls (Fig.9A, Table S1), meaning that CaMKII functions downstream of Kek-6. Together,

365   these data show that Kek-6 activates CaMKII.

366   Pre-synaptic CaMKII localizes to active zones [49,55], so next we asked whether

367   CaMKII was required for the increased active zones caused by *kek-6* gain of function.

368   CaMKII inhibition with Ala or knock-down with RNAi decreased active zones (Fig.S4A,B and

369   Fig.9B, Table S1) and over-expression of *activated CaMKII* in motoneurons increased active

370   zones *(*Fig.S4A,C, Table S1). Remarkably, over-expression of *Ala* or *CaMKII-RNAi* together

371   with *kek6*, rescued the increase in active zones caused by *kek6* gain of function (Fig.9C,D,

372   Table S1*)*. These showed that CaMKII is required downstream of Kek-6 for active zone

373   formation. Furthermore, in *kek6$^{-/-}$* mutants and *kek6$^{-/-}$ DNT2$^{-/-}$* double mutants the levels of

374   pCaMKII^{T287} decreased (Fig.10A,Table S1), showing that Kek-6 is required for CaMKII

375   activation at the NMJ. We found no significant effect of *DNT2* loss or gain of function on

376   pCaMKII^{T287} levels at the NMJ. Importantly, over-expressing activated *CaMKII^{T287D}* pre-

377   synaptically rescued the phenotypes of decreased axonal length and reduced Ib boutons of

378   *kek6$^{-/-}$* (Fig.10B) and *DNT2$^{-/-}$* (Fig.10C) single mutants, and *kek6$^{-/-}$ DNT2$^{-/-}$* double mutants

379   (Fig.10D) (Table S1). This means that the mutant phenotypes were caused, at least partly,

380   by decreased CaMKII activation. Together, these data demonstrate that Kek-6 and DNT2

381   function in concert upstream of CaMKII to regulate NMJ size and active zones.

382   To test the functional link between Kek-6, DNT2 and VAP33A, we used genetic

383   epistasis. Loss of *VAP33A* function in mutants caused a reduction in Ib boutons (Fig.11A,B),

384   and pre-synaptic over-expression of *VAP33A* increased Ib bouton number (Fig.11A,D,E,

385   Table S1), as previously reported[52]. These phenotypes were also shared with alterations in

386   *kek6* and *DNT2* levels, consistent with common functions. Importantly, over-expression of

387   *VAP33A* in motoneurons rescued bouton number in *kek-6$^{-/-}$* (Fig.11A,C) and *DNT2$^{-/-}$*

388   (Fig.11A,D) single mutants (Table S1). Thus, VAP33A functions downstream of Kek-6 and

14

389   DNT2. Interestingly, *kek-6*$^{-/-}$ *DNT2*$^{-/-}$ double mutants rescued the increase in Ib boutons

390   caused by *VAP33A* over-expression, restoring bouton number down to control levels

391   (Fig.11A,E, Table S1). This suggests that VAP33A may be required both for pre-synaptic

392   vesicle release downstream of Kek6, and post-synaptic secretion of DNT2.

393   To conclude, altogether these data show that CaMKII and VAP33A function

394   downstream of DNT2 and Kek6 in motorneurons.

395

396   DISCUSSION

397

398   Drosophila homologues of the Trk receptor family had long been sought. This was important

399   to find fundamental principles linking structure and function in any brain. Here, we show that

400   Kek-6 is a Trk-family receptor lacking a TyrK, for the neurotrophin ligand DNT2, regulating

401   structural synaptic plasticity via CaMKII and VAP33A, in *Drosophila* (Fig.12A,B).

402   Starting with an unbiased approach, we showed that of all Drosophila LIGs Keks are

403   the most likely DNT receptors in the CNS, and used two assays to demonstrate binding of

404   Kek-6 to DNTs. Using in vivo analyses, we demonstrate that motoneurons express *kek-6*,

405   and Kek-6 binds DNT2 which is produced by the muscle in post-synaptic boutons, and

406   functions retrogradely. *kek-6* and *DNT2* share mutant phenotypes, *kek6 DNT2* double

407   mutants have synergistic phenotypes, and *kek-6* over-expression can rescue mutant

408   phenotypes of *kek6*$^{-/-}$ and *DNT2*$^{-/-}$ single single and *kek6*$^{-/-}$*DNT2*$^{-/-}$ double mutants. Kek-6 is

409   not a ligand sink or dominant co-receptor for Toll-6. Instead, Kek-6 and Toll-6 can interact

410   physically and function cooperatively as DNT2 receptors. Kek-6 recruits CaMKII and

411   VAP33A to a pre-snaptic downstream complex and induces CaMKII activation. Epistasis

412   analysis demonstrated that Kek6 functions in motorneurons downstream of DNT2 and

413   upstream of CaMKII and VAP33A to promote NMJ growth, and active zone formation. We

414   conclude that DNT2 and its receptors Kek-6 and Toll-6 regulate synaptic structural plasticity

415   and homeostasis.

15

416   Despite abundant evidence that retrograde signals and positive feedback loops

417   regulate structural synaptic plasticity at the *Drosophila* glutamatergic NMJ, the identification

418   of retrograde factors had been scarce [45,56], creating a void for the general understanding

419   of structural synaptic plasticity. BDNF is a key retrograde ligand in mammals, and together

420   with its receptor TrkB they form a positive feedback loop that reinforces synaptic function

421   [1,4]. We have shown the neurotrophin DNT2 is a retrograde ligand for Kek6 (Fig.12B).

422   DNT2 is produced in muscle and over-expression of tagged DNT2 in muscle leads to its

423   distribution in pre-synaptic boutons; over-expression of *DNT2* in muscle induces bouton,

424   active zone formation and axonal terminal growth, pre-synaptically; *DNT2* mutants have

425   smaller NMJs, and pre-synaptic over-expression of either *kek6, CaMKII* or *VAP33A* can

426   rescue the *DNT2* loss of function phenotype; and conversely, *kek6* loss of function rescues

427   the phenotype caused by *DNT2* over-expression in muscle. Thus, DNT2 is an evolutionarily

428   conserved retrograde factor at the *Drosophila* synapse.

429   We demonstrate that *keks* are the *Drosophila Trk*-family homologues. Previous

430   searches for Trk-family receptors in fruitflies had focused on the Tryrosine Kinase domain,

431   and did not identify any bona fide candidates [14-16,18]. Subsequent proteomic analyses

432   confirmed the absence of full-length Trk receptors with a conserved tyrosine kinase in

433   fruitflies [19-21]. Since those earlier searches, Trks in mammals have been found to encode

434   multiple isoforms, most of which lack the tyrosine kinase domain [6,57]. Human TrkB has 100

435   isoforms, which produce 36 different proteins, of which four are abundant, and the most

436   abundant isoform in the adult human brain is truncated TrkB-T1, which lacks the tyrosine

437   kinase [6,57]. Paradoxically, the function of TrkB-T1 in neurons in the mammalian brain is

438   unknown. Our findings suggest that truncated Trk family receptors could have an

439   evolutionarily conserved function regulating structural synaptic plasticity.

440   We also show that Kek-6 functions cooperatively rather than antagonistically with pre-

441   synaptic Toll-6, a known receptor for DNT2, that is also required for synaptic structural

442   plasticity [43]. LIG proteins and truncated Trk isoforms can function as 'dominant negative'

443   co-receptors or 'ligand sinks'. TrkB-T1 can form dimers with TrkB-FL and abrogate signaling,

444   and bind BDNF rendering it unavailable to TrkB-FL [6,7]. This is thought to modulate kinase-

445   signaling levels by TrkB [58]. Similarly, in *Drosophila*, Kek-5 is an inhibitor of BMP signaling

446   [26] and Kek1 an inhibitor of EGFR signaling [59]. Thus, each Kek could function as a

447   specific inhibitor of different signaling pathways. We have shown that Kek-6 and Toll-6 can

448   form a receptor complex for DNT2, as DNT2, as well as binding Toll-6 [38], can also bind

449   Kek-6, and Kek-6 and Toll-6 interact physically. However, our evidence shows that Kek6

450   does not function as an inhibitor of Toll-6, and instead Kek-6 and Toll-6 have distinct

451   functions that positively cooperate to regulate NMJ structure and growth. All *kek-6*$^{-/-}$ and

452   *Toll-6*$^{-/-}$ single mutants, and *kek-6*$^{-/-}$ *Toll-6*$^{-/-}$ double mutants, have smaller NMJs, ruling out

453   antagonistic functions. Toll-6 is required for active zone formation, and over-expression of

454   *kek-6* also increases active zones, which means Kek-6 does not repress Toll-6. Interactions

455   between DNT2, Kek-6 and Toll-6 are required for homeostatic compensation of active zones,

456   as compensation fails in *kek6*$^{-/-}$ *DNT2*$^{-/-}$ and *kek-6*$^{-/-}$ *Toll-6*$^{-/-}$ double mutants. Our data

457   suggest that Kek-6 and Toll-6 influence synaptic structure via alternative, independent

458   pathways. Toll-6 functions in neurons via a canonical pathway to regulate NFκB and JNK

459   [36]; Toll-8 and Toll-6 regulate NMJ plasticity also via an alternative dFOXO pathway[37,43];

460   and we show that Kek-6 functions via CaMKII and VAP33A to regulate synaptic structure

461   independently of Toll-6 (Fig.12B). With our assays, DNTs can bind both Tolls [38] and Keks

462   (this work) promiscuously. The biological significance of this promiscuity is not understood,

463   as ligand-receptor interactions will depend on the availability of each one in different contexts

464   and times. Altogether, our findings strongly suggest that DNTs, Keks and Tolls constitute a

465   novel molecular mechanism that regulates synaptic structural plasticity and homeostasis.

466   We do not yet know how CaMKII gets activated downstream of Kek-6. CaMKII

467   activation and recruitment to active zones depends on the intracellular increase in Ca^{2+}

468   levels, both through Ca^{2+} channels, and Ca^{2+} stores through the PLC and IP3 pathways

469   [50,55]. In mammals, TrkB-T1 regulates Ca^{2+} levels in glia and in the heart, two contexts

470   expressing high levels of TrkB-T1 and no TrkB-FL [60-62]. However, the mechanism by

471   which TrkB-T1 raises Ca^{2+} is unknown [60,61], and whether this results in the activation of

17

472   CaMKII is unclear or may be context dependent [60,62]. CaMKII activation in neurons could

473   depend on the increase in Ca^{2+} with neuronal activity. A potential link of Kek-6 to neuronal

474   activity was revealed by the increase in ghost boutons with *kek-6* gain of function.

475   Furthermore, *kek-2* expression is modulated by neuronal activity, appropriate Kek2 levels at

476   the NMJ are required for normal synaptic structure, and DNT2 can modulate the Na+/K+

477   ATPase [27,63]. Thus, Kek-6 could function via a PLC pathway or by influencing membrane

478   channels, to increase Ca^{2+} levels and induce CaMKII activation.

479   The finding that Trk-like receptors can regulate neuronal plasticity independently of

480   kinase signalling is very important. Activation of PLCγ by TrkB TyrK signaling is generally

481   seen as a key mechanism of plasticity [1,5]. Yet CaMKII is necessary and sufficient for

482   synaptic structural plasticity, LTP and long-term memory, and has wide functions in synaptic

483   organization and homeostasis [49-51]. CaMKII can function as a frequency detector, and

484   cause long lasting changes in synaptic strength, structure and brain plasticity [50]. Thus, the

485   regulation of CaMKII and Ca^{2+} by truncated Trk-family receptors and Keks means that they

486   could regulate structural synaptic plasticity independently of the canonical TyrK-dependent

487   PLCγ pathway.

488   Keks are not identical to truncated Trk receptors and may carry out further functions

489   that could be implemented via other routes in mammals. TrkB-T1 has a very short

490   intracellular domain, whereas Keks have longer intracellular fragments including a

491   PSD95/Dlg/ZO1 (PDZ) motif [22,24,25]. PDZ domains are involved in scaffolding, and

492   assembly of post-synaptic complexes that regulate the size and strength of synapses [64].

493   Through its PDZ domain, Kek-6 might recruit synaptic partners and Ca^{2+} channels.

494   Interestingly, truncated TrkC-T1 binds the PDZ-containing protein Tamalin to induce cellular

495   protrusions via Rac[65]. Thus, it is imperative to explore further functional conservation or

496   divergence of signaling mechanisms of Keks vs. truncated Trks.

497   To conclude, evolution may have resolved how to implement structural synaptic

498   plasticity through distinct mechanisms in fruit-flies and humans, or perhaps common

499   molecular principles are shared by both, even if not in every detail. We found that in fruitflies,

500   truncated-Trk-like receptors encoded by the Keks bind neurotrophin ligands to regulate

501   structural synaptic plasticity via CaMKII and VAP33A, and the receptor complex also

502   includes Tolls. It is compelling to consider whether such a non-canonical mechanism of

503   neuronal plasticity, downstream of neurotrophins and Trk receptors but independently of

504   kinase signaling, may also operate in humans, as it could uncover novel mechanisms of

505   brain function and brain disease.

506

507   ACKNOWLEDGEMENTS

517

518   MATERIALS AND METHODS

519   **Genetics. Mutants:** *Df(3R)ED6361* lacks the kek-6 locus (Kyoto Stock Centre), *Df(3L)6092,*

520   *Df(3L)ED4342, DNT1^{41}* and *DNT2^{e03444}* are described in[35], *Df(3L)BSC578* lacks the *Toll-6*

521   locus (Bloomington). Mutant null alleles *kek6^{34}* and *kek6^{35},* and *DNT2^{37}*, were generated by

522   FRT mediated recombination of the PiggyBac insertion lines [46]: for *Kek6:*

523   *PBac{RB}kek6^{e000907}* and *PBac{WH}kek6^{f05733}*; for *DNT2: PBac{RB}spz5^{e03444}* and

524   *PBac{WH}Shab$^{f05893.}$*. Mutants were selected using genetics and PCR, using primers as

525   recommended in [46]. *kek6^{34}* is a 41.9 kb deletion that just removes the coding region for

526   *kek6;* DNT2^{37} is a 27 kb deletion that removes the ATG and first exon, most likely resulting in

527   no protein production (Foildi et al, under review). **GAL4 lines:** *kek5GAL4* line

528   *P{GawB}NP5933* (from BSC); *w;;elavGAL4* drives expression in all neurons (insertion on the

529   third chromosome); *Toll-6-GAL4* was generated by RMCE from *MIMICToll-6^{MIO2127}*;

530   *w;;D42GAL4* and *w;Toll-7GAL4* drive expression in motoneurons and have been previously

531   described[38]; *w; MhcRFP MhcGAL4* drives expression in muscle (BSC); *w; GMRGAL4*

532   drives expression in retina. **UAS lines**: to drive expression of each of the *keks* UAS-lines

533   were made as described below. *w;;UAS-DNT2-FL* expresses full length untagged DNT2;

534   *w;;UASDNT2-CK* expresses the mature form of DNT2, i.e. signal peptide+cystine-knot

535   domains; *w; UAS-DNT2-FL-GFP* expresses full-length DNT2 tagged at the COOH end, and

536   were made as described below. *w; 10xUASmyr-Td-Tomato* is membrane tethered (gift of B.

537   Pfeiffer); *w;UASCaMKII^{287D}* expresses constitutively active CaMKII and *w;UASAla* expresses

538   the CaMKII inhibitor (gifts of J. Hodge)*; UASCaMKII-RNAi: y sc v; P{y[+ v+,*

539   *TRiP.GL00237}attP2/TM3, Sb* (BSC*); y[1] w[*]; P{w[+mC]=UAS-FLAG.Vap-33-1.HA}2*

540   *(BSC).* Double mutant lines were generated by conventional genetics. **MIMIC-GFP lines:** *y*

541   *w; MIMICkek6^{MI13953}* and has a GFP insertion in the coding region (BSC). All stocks were

542   balanced over *SM6aTM6B* or *TM6B* to identify balancer chromosomes, and all were

543   generated from a *yw* or *w* mutant background. In all figures, controls are: (1) F1 from *yw x*

544   *Oregon*; (2) *w; GAL4/+*

545

546   **Molecular cloning.** Full length cDNAs for *kek1, 2, 3, 4, 5* and *6* were obtained either from

547   cDNA clones (*kek1*, *SD01674*; *kek2*, *NB7*), by PCR from cDNA libraries (gift of G. Tear; LD:

548   *kek5;* GH: *kek3*; *kek6*), or by reverse transcription PCR from larvae (*kek4*), using primers

549   designed for Gateway cloning into the pDONR plasmid: *kek1* forward:

550   GGGGACAAGTTTGTAC AAA AAA GCA GGC TCA TCC AGG AAA **ATG** CAT ATC A and

551   reverse: GGGGACCAC TTT GTA CAA GAA AGC TGG GTA GTC AGT TCT TGG TTT GGT

552   TT; *kek2*: forward: GGGGACAAGTTTGTAC AAA AAA GCA GGC TCA **ATG** AGT GGT

553   CTG CCA ATC T and reverse: GGGGACCAC TTT GTA CAA GAA AGC TGG GTA AAT

554   GTC GCT GGT TTC CTG GC; *kek3*: forward: GGGGACAAGTTTGTAC AAA AAA GCA

555   GGC TCA TAT GCG **ATG** GCA GCG GGA A and reverse: GGGGACCAC TTT GTA CAA

556   GAA AGC TGG GTA GCT CTT GAA AAT ATC CTG TC; *kek4*: forward:

557   GGGGACAAGTTTGTAC AAA AAA GCA GGC TCA CTA GAC CTT CCG TTC CTT, and

558   reverse: GGGGACCAC TTT GTA CAA GAA AGC TGG GTA TAT TGA GAT ATC AAC ACC

559   AG; *kek5*: forward: GGGGACAAGTTTGTAC AAA AAA GCA GGC TAG CTA GAC GCA

560   GAC TTA GAG and reverse: GGGGACCAC TTT GTA CAA GAA AGC TGG GTA GAC CTC

561   GGT GCC ATC CTC GC; *kek6:* forward: GGGGACAAGTTTGTAC AAA AAA GCA GGC

562   TCA **ATG** CAT CGC AGC ATG GAT C and reverse: GGGGACCAC TTT GTA CAA GAA

563   AGC TGG GTA GAG CGA CAC GAA CTC GCC AG. *CaMKII* and *VAP33A* full-length cDNAs

564   were cloned using the Gateway System first into *pDONR*, and then into *pAct5-attR-HA.*

565   For expression in flies under UAS/GAL4 control, cDNAs were subcloned to a

566   Gateway *pUASt-attR-mRFP* destination vector for conventional transgenesis, injected by

567   BestGene (www.thebestgene.com). For expression in S2 cells, they were subcloned into

568   tagged *pAct* destination vectors *pAct5c-attR-mCFP, pAct5c-attR-FLAG* and *pAct5c-attR-HA*.

569   Constructs used for expression of S2 cells of DNT1 and 2, Toll-6 and 7 were as previously

570   described[38]. Chimaeric Kek-Toll-6 receptors were generated after analyzing the domain

571   composition of the proteins using ProSite (ExPASy), PFAM, SMART, TMHMM and TMPred

572   algorithms and PubMed data. Primers were designed to amplify the sequences that encode

573   the extracellular and transmembrane domains of Kek3–6, including 15 amino acids C-

574   terminal to the Kek transmembrane region, and the intracellular domain of Toll6, at halfway

575   between the transmembrane region and the TIR domain. A unique enzyme site (BamHI or

576   EcoRI) was included in the designed primers to join the *kek* and *Toll-6* sequences, and attB

577   sites were introduced in the primers at the 5′ and 3′ ends of the chimaeric insert for Gateway

578   cloning into *pAct5c-attR-3xHA* destination vector. Unfortunately, we were not able to

579   generate *kek1-Toll-6* and *kek2-Toll-6* chimaeric receptor constructs. To clone chimaeric

580   *kek3,4,5,6-Toll-6* receptor constructs, reverse primers at the juxtamembrane of *keks* were as

581   follows: *kek3-Toll-6:* CGAT-GAATTC-AGGTACAGAGTTCCAGAGAC; *kek4-Toll-6:* CGCG-

582   GAATTC-TTGCAAATAAGTGTGCTGGC; *kek5-Toll-6:* CTAT-GGATCC-

21

583 GCTCATCATGGTGGTGTCCT; *kek6-Toll-6*: GTAT-GAATTC-

584 ACGCCGGCCTTGTTGGCATG. *Toll-6* primers from chimaeras were: Forward primers from

585 juxtamembraneCATG-GAATTC-AACTTCTGCTACAAGTCACC (compatible with *kek3*, *kek4*

586 and *kek6* chimaeras) or CATG-GGATCC-AACTTCTGCTACAAGTCACC (compatible with

587 *kek5* chimaera) and reverse from C terminus: GGGGACCAC TTT GTA CAA GAA AGC TGG

588 GTC CGC CCA CAG GTT CTT CTG CT.

589 Un-tagged full-length DNT2 was cloned into the pUASt-attB vector by conventional

590 cloning, DNT2-full length (DNT2-FL) was PCR-amplified from cDNA libraries, and cloned into

591 pUASt-attB for ΦC31 transgenesis [66]. UAS-DNT2-FL-GFP was cloned by Gateway cloning

592 into pUAS-GW-GFP, followed by conventional transgenesis.

593

594 **Luciferase signaling in S2 cells.** For Dif signaling assays using chimaeric kek-Toll-6

595 receptors, S2 cells stably transfected with *drosomycin-luciferas*e were maintained at 27^{0}C in

596 air in Insect-Xpress medium (Lonza) supplemented with penicillin/streptomycin/L-glutamine

597 mix (Lonza) and 10% foetal bovine serum (Lonza). 1ml suspended cells were passaged

598 every two days into 4ml fresh medium. 3×10^{6} cells in 2ml media were seeded per well of a 6-

599 well plate 24 hours prior to transfection. Per well of experiment, 250μl serum-free media, 3μl

600 TransIT-2020 (Mirus), 2μg of HA tagged chimaeric receptors *kek-toll6-HA* plus 1μg of *pAct-*

601 *renilla-luciferase* were incubated at room temperature for 30 minutes, supplemented with

602 350μl serum-free media and added to aspirated cells. After 4 hours, transfection mixture was

603 removed and 2ml supplemented medium added. All experiments were conducted 48 hours

604 after transfection; for imaging membrane targeting of Kek-Toll6-HA protein S2 cells were

605 starved for 6h prior to fixation.

606 For signaling assays, S2 cells were stimulated with 50nM/well of purified baculovirus

607 DNT2 protein (generated as previously described[38]) and Dif signalling quantified by

608 luminescence. DNT2 was added 48 hours after transfection and luminescence quantified 24

609 hours after DNT stimulation. Transfected and stimulated cells were pelleted from single

610 wells, resuspended in 400μl media and separated into three 50μl aliquots in an opaque 96-

22

611   well plate. 40µl of Firefly Luciferase Substrate (Dual-Glo Luciferase Assay System;

612   Promega) was added per 50µl aliquot, incubated for 10 minutes, and luminescence

613   measured using a Mithras LB 940 Multimode Microplate Reader (Berthold). 40µl Stop & Glo

614   substrate (Dual-Glo Luciferase Assay System; Promega) was added to quench Dif signal

615   and activate Renilla Luciferase. Renilla luminescence data was used to normalize Firefly

616   Luciferase data.

617

618   **Co-immunoprecipitations.** Coimmunoprecipitations were carried out as previously

619   described[38], after transfecting standard S2 cells with the following constructs: (1) Co-IP

620   Kek6-DNT2: *pAct5C-Pro-TEV6HisV5-DNT2-CK* and *pAct5C-Kek6-3xHA*; (2) Co-IP Kek6-

621   DNT2 or Kek6-DNT1: *pAct5C-Kek6-3xHA, pAct5C-Pro-TEV6HisV5-DNT1-CK-CTD, pAct5C-*

622   *Pro-TEV6HisV5-DNT2-CK;* (3) Co-IP Kek5-DNT2: *pAct5C-Kek5-3xFlag* and *pAct5C-Pro-*

623   *TEV6HisV5-DNT2-CK.* (4) Co-IP kek6-Toll-6: *pAct5C-Kek6-HA* and *pAct5C-Toll-6^{CY}-3xFlag,*

624   *pAct5C-Toll-7^{CY}-3xFlag . .*(5) Co-IP Kek6-CaMKII: *pAct5-CaMKII-HA* and *pAct-kek-6-FLAG.*

625   (6) Co-IP kek6-VAP33A*: pAct5-Vap33A-HA* and *pAct-kek-6-FLAG.* 48h after transfection

626   cells were harvested and washed in PBS. Final cell pellets were lysed in 600 µL NP-40

627   buffer (50 mM Tris-HCl pH:8.0, 150 mM NaCl, 1% Igepal-630) supplemented with protease

628   inhibitor cocktail (Pierce). For V5 and HA immuno-precipitations,500 µL of lysates from single

629   or co-transfected cells were incubated with 1 µg of mouse anti-V5 or 1 µg of mouse anti-HA

630   antibodies overnight at 4 °C, then the lysate plus antibody mixtures were supplemented with

631   25 µL of protein-A/G megnetic beads (Pierce) and incubated for 1 h at room temperature.

632   For Flag immunopreciptations, lysates were incubated with anti-Flag antibody conjugated

633   magnetic beads (Sigma-Aldrich) overnight at 4 °C. In both cases, beads were washed

634   thoroughly in NP-40 buffer and/or PBS. Proteins were eluted in 40 µL of 2x Laemmli-buffer

635   and analysed by Western blot following standard procedures.

636

637   **Pull-down assays and proteomics.** S2 cells were transfected with 4 µg *pAct5C-Kek6-*

638   *3xFlag* expression construct. For controls mock-transfected cells (i.e. no construct) were

23

639   used. After transfection cells were processed as described above for co-immunoprecipitation.

640   600 µL of cell lysates were incubated with 20 µL of anti-Flag conjugated magnetic beads

641   overnight at 4 °C. Beads were washed thoroughly in PBS, then proteins were eluted in 40 µL

642   2x Laemmli-buffer for 10 min at room temperature. Eluted proteins were loaded onto 10%

643   polyacryalamide gels. Alternatively, proteins were not eluted after overnight incubation with

644   anti-Flag beads, but they were re-incubated with whole fly (OregonR) head lysates. Here, 60

645   heads were lysed in 600 µL of NP-40 buffer. After overnight incubation at 4 °C, proteins were

646   eluted and analysed as for S2 cell lysate proteins. Thus, eluted proteins were loaded onto

647   10% polyacryalamide gels, and the gels were stained with Coomassie Blue and cut into

648   several pieces. Gel pieces were subjected to in-gel digestion with trypsin using a standard

649   protocol. Tryptic peptides were analysed by LC-MS/MS using Ultimate 3000 HPLC coupled

650   to a LTQ Orbitrap Velos ETD mass-spectrometer. Peptide separation, mass spectrometric

651   analysis and database search were carried out as specified at the University of Birmingham

652   Proteomics Facility. Candidate binding proteins were identified on these criteria: (1) Proteins

653   were accepted only if they were identified with at least two high confident peptides. (2) Mock-

654   transfected controls and Kek6-3xFlag samples were compared, and only proteins identified

655   from Kek6-3xFlag lysates or heads, but absent form controls, were considered as possible

656   interacting partners for Kek6.

657

658   **Western blotting.** Western blot was carried out following standard procedures using

659   antibodies listed here: mouse anti-V5 (1:5000, Invitrogen), mouse anti-HA (1:2000, Roche),

660   chicken anti-HA (1:2000, Aves), rabbit anti-Flag (1:2000, Sigma-Aldrich), HRP-conjugated

661   anti-mouse IgG (1:5000-1:10000, Vector), HRP-conjugated anti-chicken IgG (1:5000,

662   Jackson Immunoresearch), HRP-conjugated anti-rabbit IgG (1:1000-1:5000, Vector), mouse

663   dCaMKII (Cosmo CAC-TNL-001-CAM 1:1000), rabbit α-p-CaMKIIα (Thr 286) (Santa Cruz sc-

664   12886-R 1:1000), mouse Tubulin DM1A (Abcam ab7291 1:10000).

665

666   **NMJ dissections and preparation**

24

667   NMJ preparations were carried out according to [67]. For GFP stainings in *MhcGAL4>UAS-*

668   *DNT2-FL-GFP*, L3 larvae were placed in agar plates (2%) and left at 29°C for 90 minutes to

669   potentiate the NMJ before dissections [68]. A hundred and twenty female flies and 50 males

670   were placed in a cage with a removable agar and grape juice plate. On the second day,

671   plates were changed for new ones in the morning and evening, and discarded. On the third

672   day, the plates were changed every 1h 30min and discarded. On the fourth day, the plates

673   were changed every 1h 30min and kept in a 25°C incubator for larvae collection. The next

674   day, hatched L1 larvae from each plate were transferred to a vial, and exactly 40 larvae were

675   placed in each vial. When larvae reached L3 stage (5 days after egg laying), they were

676   dissected in low calcium saline as previously described[67], and fixed for 10 minutes in

677   Bouin's solution (HT10132 SIGMA). Samples were washed 6 times for 10 minutes in PBT

678   (0.1% of Triton in 1M PBS) to remove fixative solution and kept overnight in blocking solution

679   (10% normal goat serum in 0.1% Triton in PBS 1M). Primary antibodies were incubated

680   overnight at 4°C and samples were washed the following day 8 times for 10 minutes in PBT.

681   Secondary antibodies were incubated for 2h at room temperature and samples were washed

682   8 times for 10 minutes in PBT. Samples were mounted in Vectashield anti-bleaching medium

683   (Vector Labs) in No.0 coverslips. NMJs were analysed for muscle 6/7 only; segments A3 and

684   A4 were analysed in each larva.

685

686   **Immuno-stainings and in situ hybridisations.** Antibody stainings in embryos and S2 cells

687   were carried out following standard procedures, using the following primary antibodies at the

688   indicated dilutions: mouse anti-FasII at 1:5 (ID4, Developmental Studies Hybridoma Bank,

689   Iowa); rabbit anti-GFP 1:1000 (Molecular Probes); mouse anti-Dlg 1:20 (4F3, Developmental

690   Studies Hybridoma Bank, Iowa); rabbit anti-HRP 1:250 (Jackson Immunoresearch); mouse

691   anti-Brp 1:100 (nc82, Developmental Studies Hybridoma Bank, Iowa); rabbit anti-p-

692   CaMKII^{T287} 1:150 (Santa Cruz), detects phosphorylation at Thr286 (T287 in *Drosophila*),

693   constitutively active form; mouse plain anti-Synapsin at 1:25 (DSHB 3C11). Secondary

694   antibodies were: anti-guinea pig-Alexa 488 at 1:250 (Molecular Probes); biotynilated anti-

695   mouse at 1:300 (Jackson Labs) followed by the ABC Elite Kit (Vector Labs); biotynilated anti-

696   guinea pig at 1:300 (Jackson Labs) followed by Streptavidin-Alexa-488 at 1:400 (Molecular

697   Probes); anti-rabbit-Alexa 488 at 1:250 (Molecular Probes); anti-mouse-Alexa 488 1:250

698   (Molecular Probes); anti-rabbit-Alexa 647 at 1:250 (Molecular Probes); anti-mouse-Alexa 647

699   1:250 (Molecular Probes).

700   In situ hybridizations were carried out following standard procedures, using antisense

701   mRNA probes from 5' linearised plasmids and transcribed as follows: *lambik:* a 551

702   nucleotides fragment was cloned into pDONR with primers: forward

703   GGGGACAAGTTTGTAC AAA AAA GCA GGC TAG AAA CTA CGC ATG AGC CTG and

704   reverse GGGGACCAC TTT GTA CAA GAA AGC TGG GTA CCG CTC AAA TGT CCA CTG

705   T; then linearised with HpaI and transcribed with T7 RNA polymerase. *CG15744:* a 569

706   nucleotides fragment was cloned into pDONR with primers: forward

707   GGGGACAAGTTTGTAC AAA AAA GCA GGC TGG ATT GGA TAG CCT TGG TGA and

708   reverse GGGGACCAC TTT GTA CAA GAA AGC TGG GTT TCG CTT CCA TCT CCA TCT

709   C (linearised with HpaI, transcribed with T7); *CG16974:* a 548 nucleotides fragment was

710   cloned into pDONR with primers: forward GGGGACAAGTTTGTAC AAA AAA GCA GGC

711   TTA TAT GAA TCC CGA AGG CGC and reverse GGGGACCAC TTT GTA CAA GAA AGC

712   TGG GTT TGG GGG GAG TAG ATG GTA A (linearised with HPA1, transcribed with T7);

713   *kek1* (*SD01674*+*pOT2* cDNA clone; linearised with EcoRI, transcribed with SP6 RNA

714   polymerase); *kek2* (*NB7*+*pNB40*; HindIII; T7); *kek3* (HpaI; T7); *kek4* (*GH27420*+*pOT2*;

715   EcoRI; SP6); *kek6* (in *pDONR*, HpaI; T7); *CG15744* (in pDNOR, HpaI; T7); *CG16974* (in

716   pDNOR, HpaI; T7); *lambik* (in pDONR, HpaI; T7). Colorimetric reaction was using Alkaline

717   Phosphatase conjugated anti-DIG conjugated.

718

719   **Microscopy and Imaging.** Wide-field Nomarski optics images were taken with a Zeiss

720   Axioplan microscope, 63x lens and JVC 3CCD camera and Image Grabber graffics card

721   (Neotech) and a Zeiss AxioCam HRc camera and Zen software. Laser scanning confocal

722   microscopy was carried out using a Leica SP2 AOBS inverted confocal microscope, 40x

723 lens, at 1024 x 1024 resolution, and a Zeiss LSM710 inverted laser scanning confocal

724 microscope with a 40x lens, 1024 x 1024 resolution, 0.25μm step for anti-Brp, and 0.5 μm

725 step for the rest. Images were compiled using ImageJ, Adobe Photoshop and Illustrator.

726   For NMJ data, all images provided in the figures are projections from Z stacks; all the

727 quantitative anlyses were carried out in the raw stacks, not in projections. Muscle surface

728 area was measured from bright field images using ImageJ. Anti-HRP was used to measure

729 total terminal axonal length using ImageJ. Boutons were visualized with anti-Dlg, and total

730 boutons as well as separately Is and Ib boutons, were counted manually with the aid of the

731 ImageJ Cell Counter plug-in. Automatic quantification of active zones labeled with anti-Brp,

732 and anti-pCaMKII^{T287}, were carried out in 3D throughout the stacks of images using the

733 DeadEasy Synapse ImageJ plug-in that we previously validated [39], and anti-Synapsin was

734 analysed with a slightly modified version. DeadEasy Synapse first reduces the Poisson

735 noise, characteristic of confocal microscopy images, of each slice in the stack using a

736 median filter. Subsequently, in order to separate signal (e.g. Brp+ active zones) from the

737 background, images are segmented. Since the intensity of the staining varied within each

738 image and from image to image, the maximum entropy threshold method [69] was used to

739 find a local optimal threshold value for each pixel. For this, we used a square window of size

740 15x15, sufficient to find the local optimal threshold around each pixel in an image. In this

741 way, each pixel is considered part of an active zone if the value of the pixel is higher than the

742 local threshold, otherwise assigned to the background. Since this method is computationally

743 expensive and very low intensity pixels correspond to background, it was possible to reduce

744 the computation time by assigning pixels whose intensity was lower or equal than 20 directly

745 as part of the background and only applying the thresholding method to pixels whose

746 intensity was higher than 20. Finally the volume of the active zones is measured. This

747 method worked just as accurately with Brp, pCaMKIIT^{287} and Synapsin stainings. Data were

748 normalized to muscle surface area or axonal length.

749

750   **Locomotion assays.** L3 wandering larvae were placed one at a time on an agar plate (2%)

751   and left it crawl for 40 seconds. Larvae were filmed crawling across the agar plate and then

752   discarded. Plates were cleared before placing another larva. At least 50 larvae were filmed

753   per genotype, and the test was always done in the morning. Films of 400 frames per larva

754   were analysed using FlyTracker software developed in our lab to obtain the trajectory and

755   speed, as previously described[38].

756

757   **Statistical analysis.** Data were analysed in SPSS Statistics 21 (IBM) and GraphPad Prism

758   6. Confidence interval was 95% (p<0.05). Categorical data were tested using χ^2, and a

759   Bonferroni correction was applied for multiple comparisons. Continuous data were first tested

760   for normality by determining the kurtosis and skewness, and a Levene's Test was applied to

761   test for homogeneity of variance. Data were considered not normally distributed if absolute

762   kurtosis and skewness values for each genotype were greater than 1.96 x standard error of

763   kurtosis/skewness. Variance of the populations of different samples were considered

764   unequal if Levene's test for homogeneity of variance gave a p value of <0.05. If samples

765   were normally distributed and variances were equal, Student-t tests were applied for 2-

766   sample type graphs, and One-Way ANOVA was used to compare means from >2 samples.

767   When data were normally distributed, but variances were unequal, Welch ANOVA was used

768   instead. Multiple comparison corrections to normal data were applied using a post-hoc

769   Dunnett test of comparisons to a control, Games-Howell or Bonferroni post-hoc comparing all

770   samples. Non-parametric continuous data were compared using a Mann-Whitney U-test

771   when 2 sample types were being analysed, and a Kruskal-Wallis test for >2 samples, and

772   multiple comparison corrections were applied using a post-hoc Dunn test. See Table S1 for

773   all statistical sample sizes, applied tests and p values.

774

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28

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963

964   FIGURE LEGENDS

965

966 **Fig.1 Keks are Trk-like receptors expressed in the CNS.** (A) Modular composition of TrkB,

967 TrkB-T1, Dror, Otk and *Drosophila* LIGs. (B) Amongst the LIGs, Keks are closer to the Trks

968 than any other mammalian or Drosophila LIGs, adapted from the phylogeny of Mandai et

969 al.[22]. (C-D) mRNA distribution in embryos: (C) *CG15744, lambik* and *CG16974* are not

970 expressed in the VNC (arrows) above background, but *lambik* is in PNS and *CG16974* in

971 muscle precursors (arrowheads); (D) *kek-1, kek-2* and *kek-6* transcripts are found in the

972 VNC, and *kek5GAL4>tdTomato* drives expression in VNC and PNS (right) neurons. (E)

973 Over-expression of *keks* – most prominently *kek2* and *6* - in all neurons with *elavGAL4*

974 rescued the cold semi-lethality of $DNT1^{41}$ $DNT2^{e03444}$ double mutants. Chi-square and

975 Bonferroni multiple comparisons correction. *p<0.05, ***p<0.001. For statistical details see

976 Table S1.

977

978 **Fig.2   Keks bind DNTs.** (A) Diagram of Chimaeric Kek-Toll-6-HA receptors, bearing the

979 extracellular and transmemebrante domains of Keks and the intracellular signaling domain of

980 Toll-6 (left). Chimaeric receptors visualized with anti-HA antibodies are distributed to the cell

981 membrane in S2 cells (middle). Binding of purified mature DNT2-CK to the extracellular

982 domain of Kek-Toll-6 receptors activates the *drosomycin-luciferase* reporter (right). Black

983 asterisks denote comparisons to empty vector (pDONR) control, and red asterisks refer to

984 no-stimulation controls. Welch ANOVA p<0.001; asterisks refer to post-hoc Games-Howell

985 *p<0.05, **p<0.01, ***p<0.001. (B-D) Western blots showing co-immunoprecipitations from

986 co-transfected S2 cells: (B) precipitation of Kek6-HA with anti-HA brings down DNT2-V5

987 detected with anti-V5. (C) Reverse co-IP for Kek6 and DNT2. Precipitation of DNT1-V5 also

988 brings down Kek6-HA. (D) Precipitation of DNT2-V5 brings down Kek5-Flag. Black asterisks

989 indicate the relevant control bands, red arrows indicate co-IPs. Input controls: cell lysate from

990 co-transfected cells express both proteins. IP: immunoprecipitation; WB: western blot; FL: full

991 length; CK: cystine-knot cleaved form. DNTs can be spontaneously cleaved following

992 expression from these constructs in S2 cells.

993

Fig.3 Kek-6 is expressed pre-synaptically in motoneurons and binds post-synaptic DNT2. (A-B) Kek-6^{GFP} GFP colocalises with the neuronal markers Eve (A) and HB9 (B) in the larval VNC (arrows show examples). (C) Kek-6^{GFP} was found in third instar larval muscle 6/7 NMJ and synaptic boutons (higher magnification). (D) Kek-6^{GFP} was found in the motorneuron axonal terminal (arrows), and in pre-synaptic bouton lumen (higher magnification on the right), not colocalising with the post-synaptic marker anti-Dlg (arrows). (E) Illustration. (F-J) Over-expression of GFP tagged full-length DNT2 in muscle *(MhcGAL4>UAS-DNT2-FL-GFP)* revealed: (F) DNT2-GFP distribution within the pre-synaptic bouton lumen (arrows), boutons labeled post-synaptically with anti-Dlg; (G-J) DNT2-GFP along the motoraxon and within the pre-synaptic bouton lumen, both labeled with FasII (arrows).

1005

Fig. 4 *kek6* and *DNT2* mutants have smaller NMJs. (A) Plotted trajectories of filmed larvae (left), and histograms (right) of frames vs. speed analysed with FlyTracker. Kruskal-Wallis p<0.0001 and ***p<0.001 post-hoc Dunn test. (B) NMJs (left) and box-plot graphs (right) showing: *kek-6*$^{-/-}$ and *DNT2*$^{-/-}$ single mutants and *kek-6*$^{-/-}$ *DNT2*$^{-/-}$ double mutants have fewer Dlg+ boutons and smaller HRP+ axonal terminals (normalized to muscle area, MSA). Dlg: Kruskal-Wallis p<0.0001, and *p<0.05, **p<0.01, ***p<0.001 post-hoc Dunn; HRP: One Way ANOVA p<0.0001, and **p<0.01, ***p<0.001 post-hoc Dunnett. *kek-6* and *DNT2* single mutants, but not the double mutants, have increased active zone density (Brp+/HRP+axonal length). Brp: Kruskal-Wallis p=0.0012, and **p<0.01, ***p<0.001 Dunn's post-hoc. *Kek-6* mutants have reduced Synapsin, Mann-Whitney U test ***p<0.001. For statistical details, see Table S1. Mutant genotypes throughout figures: Control: *yw/+;* Mutants: *kek-6*$^{-/-}$: *kek6*^{34}/*Df(3R)6361; DNT2*$^{-/-}$: *DNT2*^{37}/*Df(3L)6092; kek-6*$^{-/-}$*DNT2*$^{-/-}$: *kek6*^{34}*Df(3L)6092/ Df(3R)6361 DNT2*^{37}.

1019

1020 **Fig. 5 Kek-6 and DNT2 can induce active zones and NMJ growth.** Confocal images of

1021 NMJs from A3-4 muscle 6/7 (left), and box-plot graphs (right), showing: (A) Over-expression

1022 of *kek6* in motoneurons had not effect on HRP+ NMJ size, but it increased Brp+ active

1023 zones. HRP: Student t test n.s. p=0.07; Brp: Mann-Whitney U test ***p<0.001.(B) Over-

1024 expression of full-length DNT2 in muscle increased NMJ size (HRP) and active zones (Brp),

1025 revealing a retrograde function. HRP: Mann-Whitney U test *p<0.05; Brp: Student t test

1026 **p<0.01. (C) Over-expression of both full-length DNT2 and mature DNT2-CK in

1027 motoneurons induced active zone formation. Brp DNT2-CK: Student t test **p<0.01, and Brp

1028 DNT2-FL: Mann-Whitney U test ***p<0.001. See Table S1. *MN=D42GAL4.*

1029

1030 **Fig. 6 Kek-6 functions downstream of DNT2.** Confocal images of NMJs from A3-4 muscle

1031 6/7 (left), and box-plot graphs (right), showing: (A) Over-expression of *kek-6* in motoneurons

1032 rescued the phenotypes of *kek-6* mutants. Dlg: One Way ANOVA p<0.0001 and post-hoc

1033 Bonferroni *p<0.05, ***p<0.001. HRP: One Way ANOVA p<0.001 and post-hoc Bonferroni

1034 **p<0.01, **p<0.01. (B) Left: Over-expression of *kek-6* in neurons rescued the phenotype of

1035 *DNT2* mutants. Dlg: Kruskal-Wallis p=0.001, and post-hoc Dunn test **p<0.01, ***p<0.001.

1036 Right: *kek-6* loss of function rescued the increase in boutons caused by the muscle over-

1037 expression of *DNT2*. Dlg: Welch ANOVA p<0.01 and post-hoc Games-Howell *p<0.05,

1038 **p<0.01. (C) Over-expression of *kek-6* in motoneurons rescued the phenotypes of *kek-6*

1039 *DNT-2* double mutants. Dlg: Kruskal-Wallis p=0.001 and post-hoc Dunn's test *p<0.05,

1040 **p<0.01. HRP: Welch ANOVA p=0.000, post-hoc Games Howell **p<0.01. See Table S1.

1041 GAL4 drivers: Muscle: *MhcGAL4*; Neurons: *elavGAL4;* MN: *D42 or Toll-7GAL4.* Controls:

1042 white boxes: yw/+; grey boxes: GAL4/+; mutant genotypes as in Fig.4. Rescue genotypes:

1043 (A) *w; UASkek6RFP/+; Df(3R)6361/kek6^{34}D42GAL4.* (B) *w; UASkek6RFP/+; elavGAL4*

1044 *Df(3L)6092/ DNT2^{37}* ; and *w; UASDNT2-FL/+; Df(3R)6361/kek6^{34}D42GAL4.* (C) *w; Toll-*

1045 *7GAL4/UASkek6RFP; kek6^{34}Df(3L)6092/ Df(3R)6361 DNT2^{37}.*

1046

1047 **Fig.7   Kek-6 and Toll-6 interact for NMJ structural homeostasis.** (A) *Toll-6GAL4>mCD8-*

1048 *GFP* revealed expression in motorneurons at the muscle 6/7 NMJ, colocalising with FasII

1049 presynaptically. (B) Muscle 6/7 NMJs (left) and box-plot graphs (right) showing: *Toll-*

1050 *6^{MIO2127}/Df(3L)BSC578* mutants and *Toll-6^{MIO2127}Df(3R)6361/kek6^{35} Df(3L)BSC578* double

1051 mutants had smaller NMJs (HRP, Kruskal-Wallis p=0.0001), and reduced active zones (Brp,

1052 Kruskal-Wallis p=0.0055), post-hoc Dunn for both *p<0.05, ***p<0.001. (C) Co-

1053 immunoprecitation from co-transfected S2 cells: Precipitating Toll-6 and Toll-7 with anti-Flag

1054 brought down Kek-6 detected with anti-HA. IP: immuno-precipitation; WB: western blot;

1055 asterisk: co-IP. See Table S1.

1056

1057 **Fig.8   Kek-6 physically interacts with synaptic factors.** (A) Diagram of pull-down

1058 workflow and (B) coomassie-stained gel showing proteins from S2 cell or adult fly head

1059 lysate bound to Kek-6-Flag or mock control. Red boxes indicate fractions used for mass

1060 spectrometry. Hits that appeared in mock controls, or with only one peptide, were dis-

1061 regarded. (C) Selection of candidate interacting proteins, see Tables S2 and S3. (D) Co-

1062 immunoprecipitations from co-transfected S2 cells: precipitating Kek-6-Flag brought down

1063 CaMKII-HA and VAP33A-HA. IP: immunoprecipitation, WB: western blot. (E) Western blots

1064 with anti-activated pCaMKII^{T287}, non-phosphorylated plain CaMKII and Tubulin, from adult fly

1065 heads, GMRGAL4 drives expression in retina. Graphs show pCaMKII^{T287} levels normalized

1066 over non-phosphorylated CaMKII. Student paired t-tests. *p<0.05, **p<0.01, see Table S1.

1067

1068 **Fig. 9   Kek6 activates CaMKII at the NMJ.** NMJs from A3-4 muscle 6/7 (left), and box-plot

1069 graphs (right), NMJs, labeled with anti-pCaMKII^{T287} for the constitutively active form; anti-Dlg

1070 for post-synaptic boutons; anti-HRP for pre-synaptic axonal terminal length; anti-Brp for

1071 active zones. Brp and pCaMKII^{T287} were quantified automatically with DeadEasy Synapse.

1072 (A, B) Over-expression of *kek-6* in motoneurons increased pCaMKII^{T287} levels, which was

1073 rescued with the over-expression of the CaMKII inhibitor Ala (A) *or* CaMKII RNAi knock-down

1074 (B) pre-synaptically. Kruskal-Wallis p<0.0001 and **p<0.01, ***p<0.001post-hoc Dunn for

1075   both graphs. (C,D) CaMKII inhibition with Ala (C) or knock-down with CaMKII-RNAi (D)

1076   rescued the increase in active zones caused by *kek-6* over-expression. (C) Welch ANOVA

1077   p<0.000 and ***p<0.001 post-hoc Games-Howell, (D) Kurskal-Wallis p<0.0001 and **p<0.01,

1078   ***p<0.001 post-hoc Dunn's. See Table S1. MN=motoneurons. Genotypes: (A-D) Control*: w;*

1079   *D42GAL4/+.* (A, C) *w; D42GAL4/UASkek6RFP. w; D42GAL4/UASAla; w;*

1080   *D42GAL4/UASkek6RFP UASAla.* (B, D) *D42GAL4/UASCaMKIIRNAi. w;*

1081   *D42GAL4/UASkek6RFP UASCaMKIIRNA*i.

1082

1083   **Fig. 10 CaMKII functions downstream of Kek6 and DNT2 at the NMJ.** Confocal images

1084   showing A3-4 muscle 6/7 NMJs, labeled as in Figure 9. (A) Reduced pCaMKII^{T287} levels in

1085   *kek-6* mutants, and *kek-6 DNT2* double mutants. Kurskal-Wallis p=0.0009, and post-hoc

1086   Dunn. (B-D) Over-expresison of activated *CaMKII^{T287D}* in motoneurons rescued the NMJ

1087   phenotypes of (B) *kek-6* mutants, Kruskal-Wallis p=0.0081, post-hoc Dunn; (C) *DNT2*

1088   mutants, Kruskal-Wallis p=0.0001, post-hoc Dunn, and (D) *kek-6 DNT2* double mutants .

1089   Welch ANOVA p<0.000, post-hoc Games-Howell. *p<0.05, **p<0.01, ***p<0.001 see Table

1090   S1. Genotypes: MN=motoneurons, *D42GAL4;* Neurons: *elavGAL4.* Different neuronal drivers

1091   were used due to genetic constraints. Control: wild-type: *yw/+;* grey boxes: *D42GAL4/+.* (A)

1092   Mutant genotypes as in Fig.4; Rescues: (B) *w;UASCaMKII^{T287}/+; D42GAL4 kek6^{34}/*

1093   *Df(3R)6361.* (C) *w;UASCaMKII^{T287D}/+; elavGAL4 Df(3L)6092/DNT2^{37}.* (D) *w;*

1094   *UASCaMKII^{T287D}/Toll-7GAL4; kek6^{34}Df(3L)6092/ Df(3R)6361 DNT2^{37}.*

1095

1096   **Fig. 11 VAP33A functions downstream of Kek-6.** (A) Confocal images of NMJs from

1097   segments A3-4, muscle 6/7. (B-E) Box-plot graphs. (A) *VAP33A^{G0231}* mutants have reduced

1098   bouton number, Mann-Whitney U test ***p<0.001. (C,D) Pre-synaptic over-expression of

1099   *VAP33A* rescues bouton number in (C) *kek-6* mutants and (D) *DNT2* single mutants,

1100   Kruskal-Wallis p<0.0001 and *p<0.05, ***p<0.001 post-hoc Dunn for both. (E) *kek-6 DNT2*

1101   double mutants rescue the bouton number phenotype caused by *VAP33A* gain of function,

1102   Kruskal-Wallis p<0.0001 and **p<0.01, ***p<0.001 post-hoc Dunn. See Table S1.

1103   MN=motoneuron, *D42GAL4* (D) or *Toll-7GAL4* (E). Rescue genotypes: (C) *UASVAP33A;*

1104   *D42GAL4 kek6^{34}/Df(3R)6361.* (D) UASVAP33A/+; *elavGAL4 Df(3L)6092/DNT2$^{37.}$.* (E)

1105   *UASVAP33A/Toll-7GAL4; kek6^{34}Df(3L)6092/ Df(3R)6361 DNT2^{37}*

1106

1107   **Fig.12   Retrograde DNT2 binds pre-synaptic Kek-6 activating CaMKII and regulating**

1108   **structural synaptic plasticity.** (A) Illustration of Kek-6 compared to Trk isoforms, and

1109   showing that DNT2 binds Kek-6, which functions via CaMKII and VAP33A downstream. (B)

1110   Pre-synaptic motorneuron terminal at the NMJ: DNT2 is produced at the muscle and

1111   secreted, binds pre-synaptic Kek-6, functioning via CaMKII and VAP33A downstream to

1112   regulate NMJ growth and active zones. DNT2 also binds Toll-6 and interactions between

1113   Kek-6 and Toll-6 regulate NMJ structural homeostasis.

1114

1115   SUPPORTING INFORMATION

1116   **Fig. S1   Kek-6 is expressed is not expressed in glia.** Confocal images of the VNC

1117   neuropile of third instar Kek-6$^{MIMIC-GFP}$ larvae, stained with anti-GFP and the pan-glial nuclear

1118   marker anti-Repo. Repo does not colocalise with GFP in any cells, arrows point to examples

1119   of Repo+, GFP-negative cells.

1120

1121   **Fig. S2   Altered *kek-6* function affects motoraxon targeting at embryonic NMJ.**

1122   The motoneuron marker FasII reveals motoraxon targeting phenotypes at muscle 6,7,12,13

1123   in stage 17 embryos, in *kek-6* mutants and upon over-expression of *kek-6* in all neurons (with

1124   *elavGAL4*). Arrows indicate stereotypic projections in wild-type, and mistargeting in other

1125   genotypes. Chi-square p<0.0001 and ***p<0.001 Boferroni corrections, see Table S1.

1126

1127   **Fig. S3   *kek-6* over-expression induces ghost boutons.** (A,B) Over-expression of

1128   *kek-6* in motorneurons (MN) with *D42GAL4* did not affect bouton number (Dlg, Mann-

1129   Whitney U-test not significant). (C-E) Over-expression of *kek-6* induced pre-synaptic ghost

1130   boutons lacking a post-synaptic component (arrows: HRP+, presynaptic and Dlg-negative,

1131 post-synaptic), (D) higher magnification; (E) quantification. Both bouton number and area

1132 increased, albeit not significantly. Mann-Whitney U-tests. See Table S1. *Genotypes:* Control:

1133 *D42GAL4/+.* : *kek-6$^{-/-}$*: *kek6^{34}/Df(3R)6361; MN>kek6: D42GAL4>UAS-kek6-RFP* (with one or

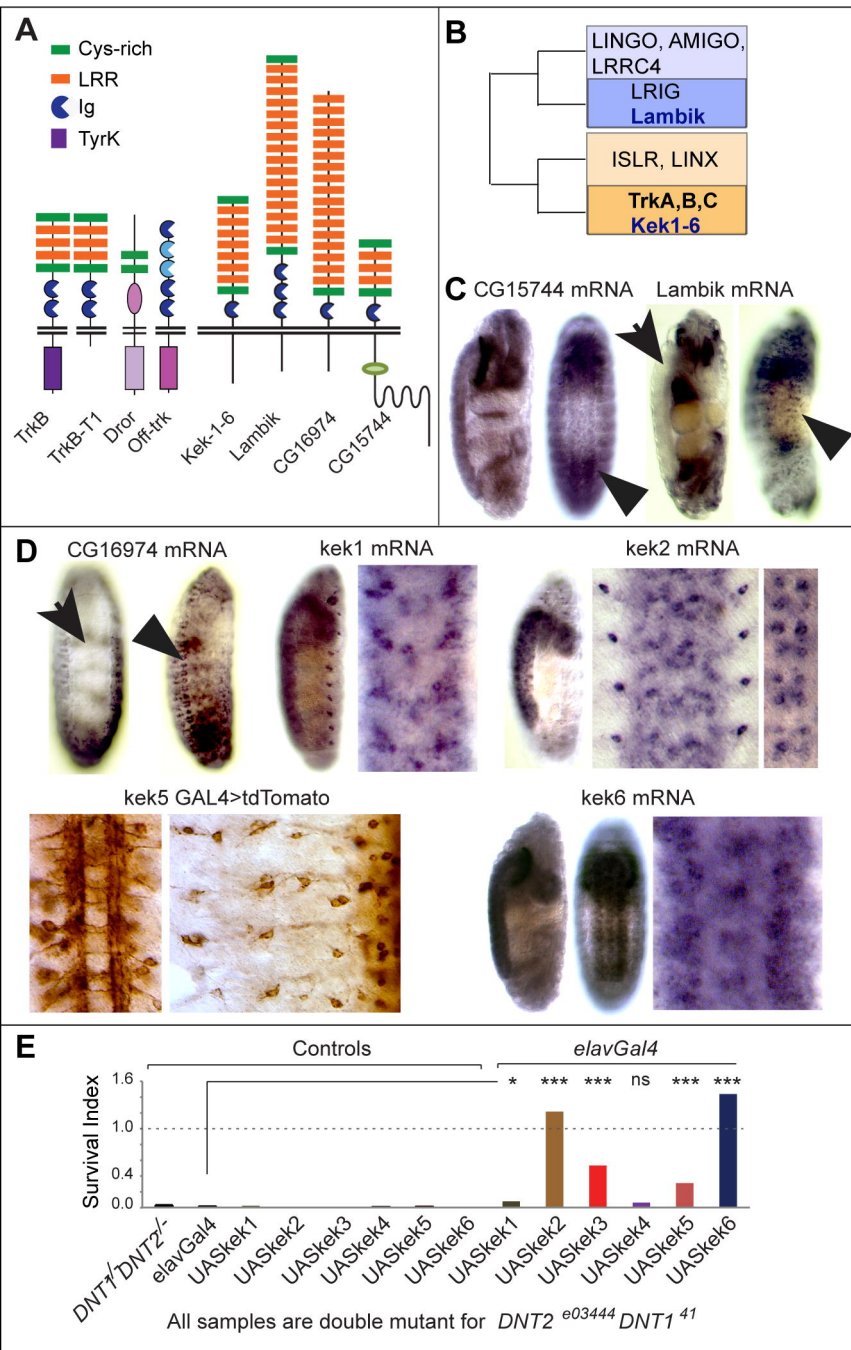1134 two copies of *UAS-kek-6*).

1135

1136 **Fig. S4   Loss and gain of CaMKII function affect the NMJ.** (A) Confocal images of

1137 muscle 6/7 NMJs, in A3-4, labeled with anti-HRP for the pre-synaptic terminal, anti-Brp for

1138 active zones, anti-Dlg for post-synaptic boutons and anti-pCaMKII^{T287D} for the constitutively

1139 active form. (B,C) Quantification. (A,B) Inhibiting CaMKII function with Ala in motoneurons

1140 (*D42GAL4>UASAla*) decreased NMJ terminal axonal length (HRP,t-test), and active zones.

1141 Student t-tests, p<0.005, **p<0.01. (A,C) Pre-synaptic over-expression of constitutively active

1142 CaMKII (*D42GAL4>UASCaMKII^{T287D}*) increased pCaMKII levels (pCaMKII^{T287D}, Student t-test

1143 **p<0.001), axonal length (HRP, Student t-test **p<0.001), Ib bouton number (Dlg, Mann-

1144 Whitney U-test ***p<0.001), and active zones (Brp, Mann-Whitney U-test **p<0.01). See

1145 Table S1.

1146

1147 **Table S1   Statistical Analysis Details.** For all genotypes, sample sizes, tests and p

1148 values, see this table.

1149

1150 **Table S2   Kek6 pull-down from S2 cells.** List of S2 cell lysate proteins binding Kek6-

1151 Flag, but not mock-transfection controls, and detected with more than one peptide.

1152

1153 **Table S3   Kek6 pull down from adult fly heads.** List of adult fly head proteins binding

1154 Kek6-Flag, but not mock controls, and detected with more than one peptide.

Figure 1   Keks are Trk-like receptors expressed in the CNS

A

Cys-rich (green)
LRR (orange)
Ig (blue)
TyrK (purple)

TrkB, TrkB-T1, Dror, Off-trk, Kek-1-6, Lambik, CG16974, CG15744

B

LINGO, AMIGO, LRRC4
LRIG
Lambik
ISLR, LINX
TrkA,B,C
Kek1-6

C   CG15744 mRNA   Lambik mRNA

D   CG16974 mRNA   kek1 mRNA   kek2 mRNA

kek5 GAL4>tdTomato   kek6 mRNA

E

Survival Index

Controls   elavGal4

* *** *** ns *** ***

1.6
1.0
0.4

DNT1/DNT2/-, elavGal4, UASkek1, UASkek2, UASkek3, UASkek4, UASkek5, UASkek6, UASkek1, UASkek2, UASkek3, UASkek4, UASkek5, UASkek6

All samples are double mutant for *DNT2* ^{e03444} *DNT1* ^{41}

Figure 2   Keks bind DNTs

A   DNT2 induces signalling from chimaeric receptors
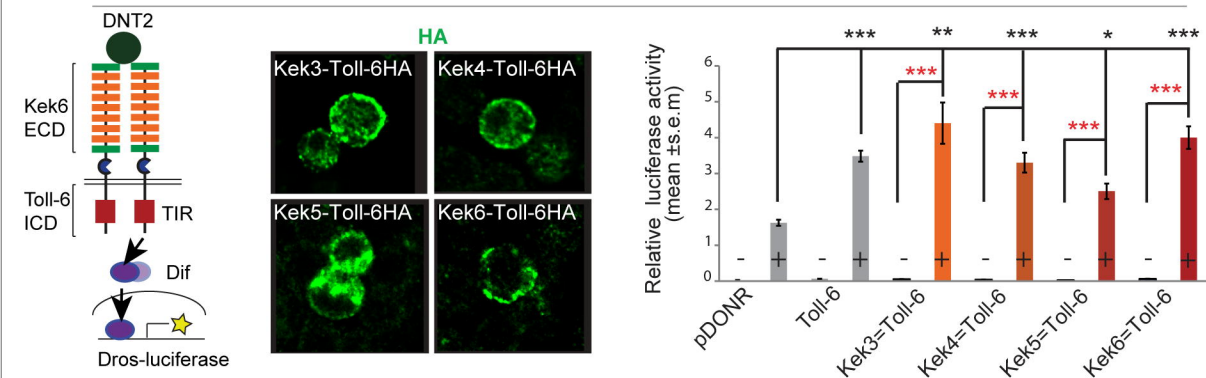
B   Co-IP Kek6-DNT2
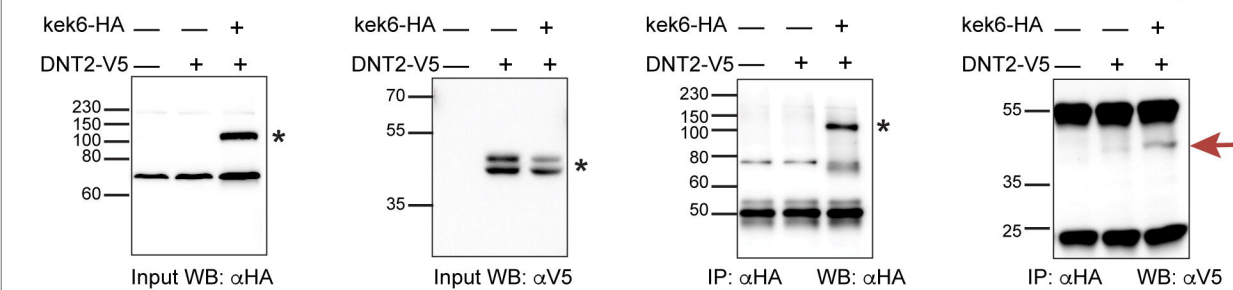
C   Reverse co-IP Kek6-DNT2, Co-IP Kek6-DNT1
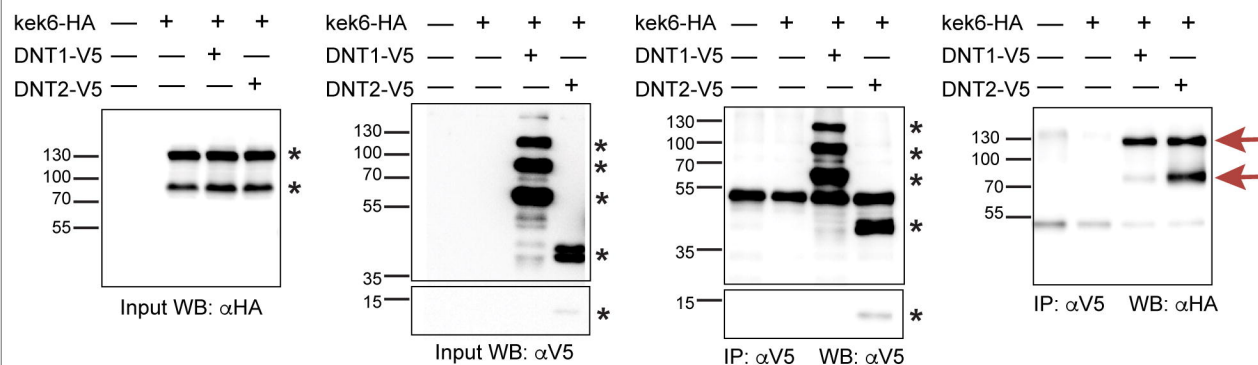
D   Co-IP Kek5-DNT2
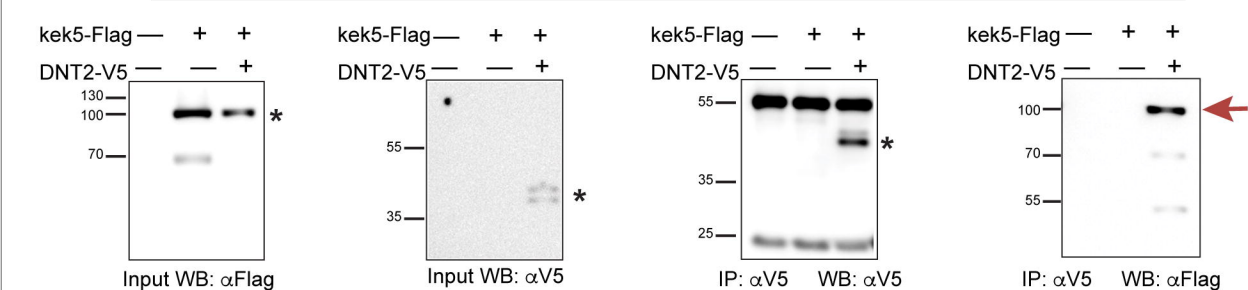
Figure 3   Kek6 is expressed pre-synaptically in motoneurons and binds post-synaptic DNT2

Figure 4   *kek6* and *DNT2* mutants have smaller NMJs

A   Larval locomotion

wild-type control
kek6–/–
DNT2–/–
kek6–/– DNT2–/–

B

Dlg: Ib boutons

HRP: Axonal length

Brp: active zones

Syn: vesicle release

wild-type control
kek6–/–
DNT2–/–
kek6–/– DNT2–/–

Figure 5   Kek-6 and DNT2 can induce active zones and NMJ growth

A *kek-6* over-expression in motoneurons

HRP: Axonal length

Brp: Active zones

Control: GAL4/+   MN>kek6

B *DNT2* over-expression in muscle

HRP: Axonal length

Brp: Active zones

Control: GAL4/+   Muscle>DNT2-FL

C *DNT2* over-expression in motoneurons

Brp: Active zones

Control: GAL4/+   MN>DNT2-CK   MN>DNT2-FL

Figure 6   Kek-6 functions dowsntream of DNT2

A

HRP   Dlg

Rescue of *kek-6* mutants

Dlg: Ib boutons   HRP: Axonal length

□ wild-type control   ■ *kek6–/–*   ■ Rescue: *kek6–/–, MN>kek6*

B

Dlg

Rescue of *DNT2* mutants   Rescue of *DNT2* GOF

□ wild-type control   ■ Control: *GAL4/+*   ■ Rescue: *DNT2–/–, Neurons>kek6*
■ *DNT2–/–*   ■ *Muscle>DNT2-FL*   ■ Rescue: *kek6–/–, Muscle>DNT2-FL*
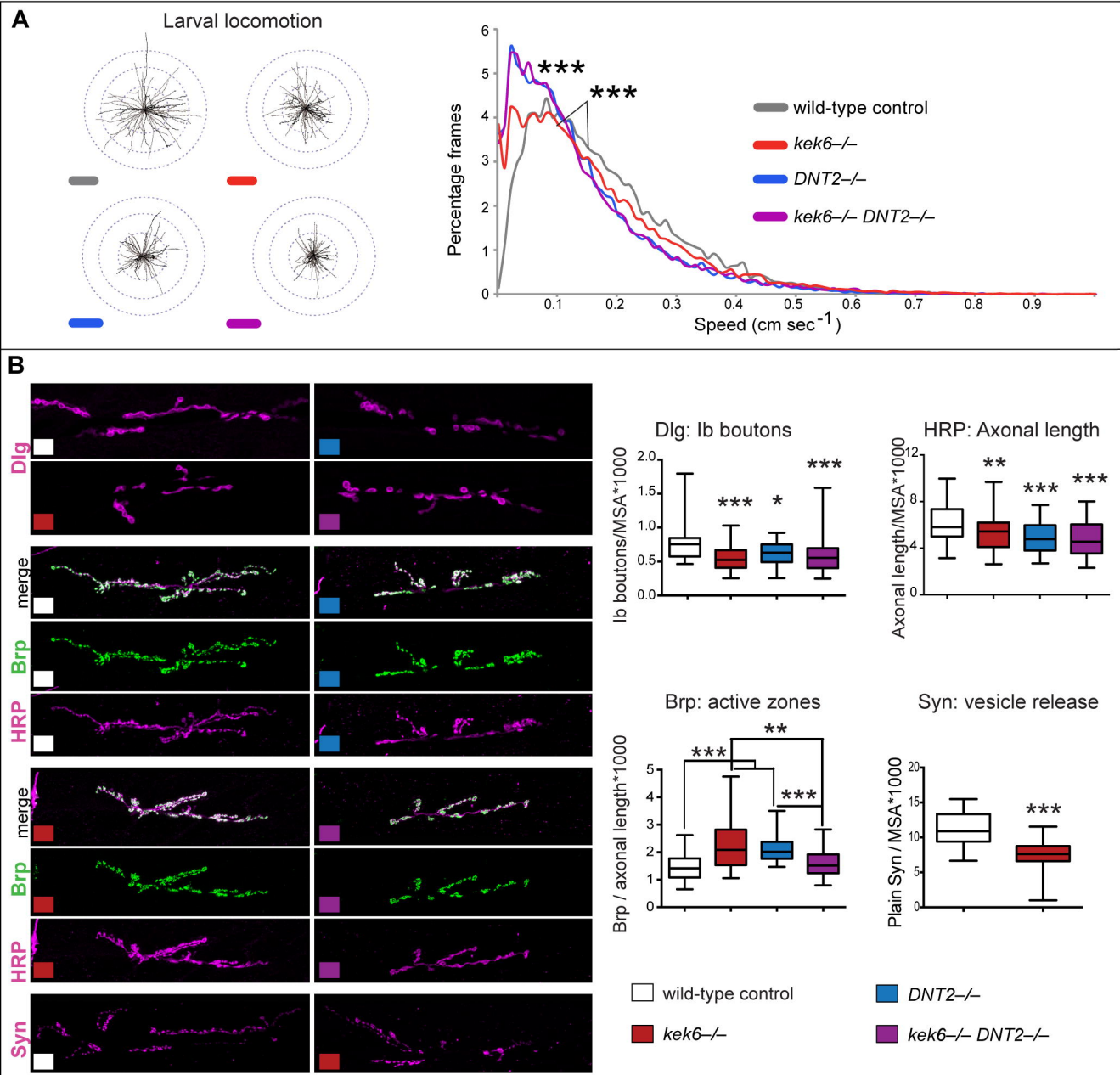
C

Rescue

merge

Dlg

HRP

Rescue of *kek-6 DNT2* double mutants

Ib / MSA * 1000   Axonal length / MSA*1000

□ wild-type control   ■ Control: *GAL4/+*   ■ Rescue:
■ *kek6-/- DNT2-/-*   ■ *MN>Kek6RFP*   *kek6-/- DNT2-/-, MN>kek6*

Figure 7   Kek-6 and Toll-6 interact for NMJ structural homeostasis.

A

| merge | FasII Toll-6>GFP | Toll-6>GFP | FasII |

B

| merge | HRP Brp | Brp |

HRP: Axonal length

Brp: Active zones

☐ wild-type control   ☐ *Toll-6–/–*   ☐ *Toll-6–/– kek6–/–*

C

Co-IP Kek-6 with Toll-6, Toll-7

Kek6-HA	—	+	+	+
Toll6^{CY}Flag	—	—	+	—
Toll7^{CY}Flag	—	—	—	+

Input: αFlag   Input: αHA

Kek6-HA	—	+	+	+
Toll6^{CY}Flag	—	—	+	—
Toll7^{CY}Flag	—	—	—	+

IP: αFlag WB: αFlag   IP: αFlag WB: αHA
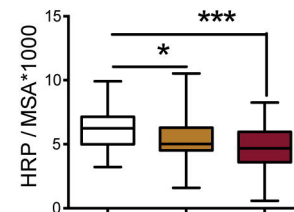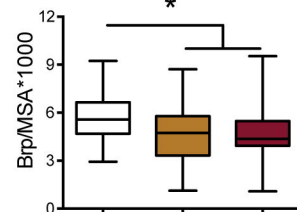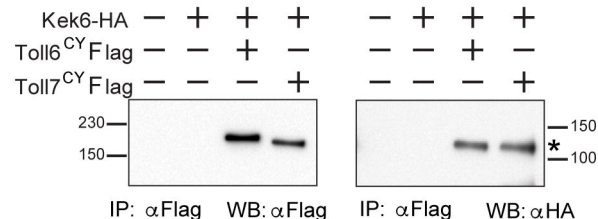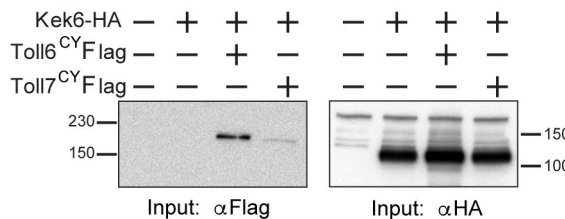
Figure 8   Kek6 physically interacts with synaptic factors.

A — Kek-6 pull-down from S2 cells and fly heads

B

C — not in controls & >1 peptide

Description	Score	Coverage	Flybase ID	GO biological process and molecular function
Calcium/calmodulin-dependent protein kinase II	20.78	14.69	FBgn0264607	learning and long-term memory, synaptic growth & transmission
Fat body protein 1, isoform B	19.06	10.23	FBgn0000639	protein transporter activity
nucleosome remodeling factor - 38kD, isoform B	18.31	26.21	FBgn0016687	transcription, ecdysone receptor signalin, Notch signaling
yolk protein 3	15.23	16.67	FBgn0004047	embryo development, neurogenesis
protein phosphatase 1 at 87B	13.08	19.21	FBgn0004103	behavior, axon guidance, CNS development, visual learning
upheld, isoform D	13.06	16.99	FBgn0004169	tropomyosin binding
Vap-33-1, isoform A	12.42	25.53	FBgn0029687	neurotransmitter secretion, synaptic maturation

D — Co-IP Kek-6 + CaMKII and Kek-6 + VAP33A

E — Kek-6 induces CaMKII activation in fly heads

$pCaMKII^{T287D}$/plainCaMKII

$pCaMKII^{T287D}$/plainCaMKII

$pCaMKII^{T287D}$/plainCaMKII

Fold change $pCaMKIIT287D$/plainCaMKII

wild type control
kek6–/–
GMR>kek6, kek6
GMRGAL4/+
GMR>DNT2-FL
Control

Figure 9   Kek6 activates CaMKII at the NMJ

Figure 10   CaMKII functions downstream of Kek6 and DNT2 at the NMJ

A pT287-CaMKII

pCaMKII^{T287}

wild type control
kek6–/–
DNT2–/–
kek6–/– DNT2–/–

B Dlg   Rescue of kek-6 mutants with activated CaMKII

Dlg: Ib boutons

Control
kek6–/–
Rescue: MN>CaMKII,^{T287D} kek6–/–

C HRP   Rescue of DNT2 mutants with activated CaMKII

HRP: axonal terminal

wild type control
Control: GAL4/+
DNT2–/–
MN>CaMKII^{T287D}
Rescue: DNT2-/-, Neurons>CaMKII^{T287D}

D HRP   Rescue of kek6 DNT2 double mutants with activated CaMKII

HRP: axonal terminal

wild type control
kek6–/– DNT2–/–
Control: GAL4/+
MN>CaMKII^{T287D}
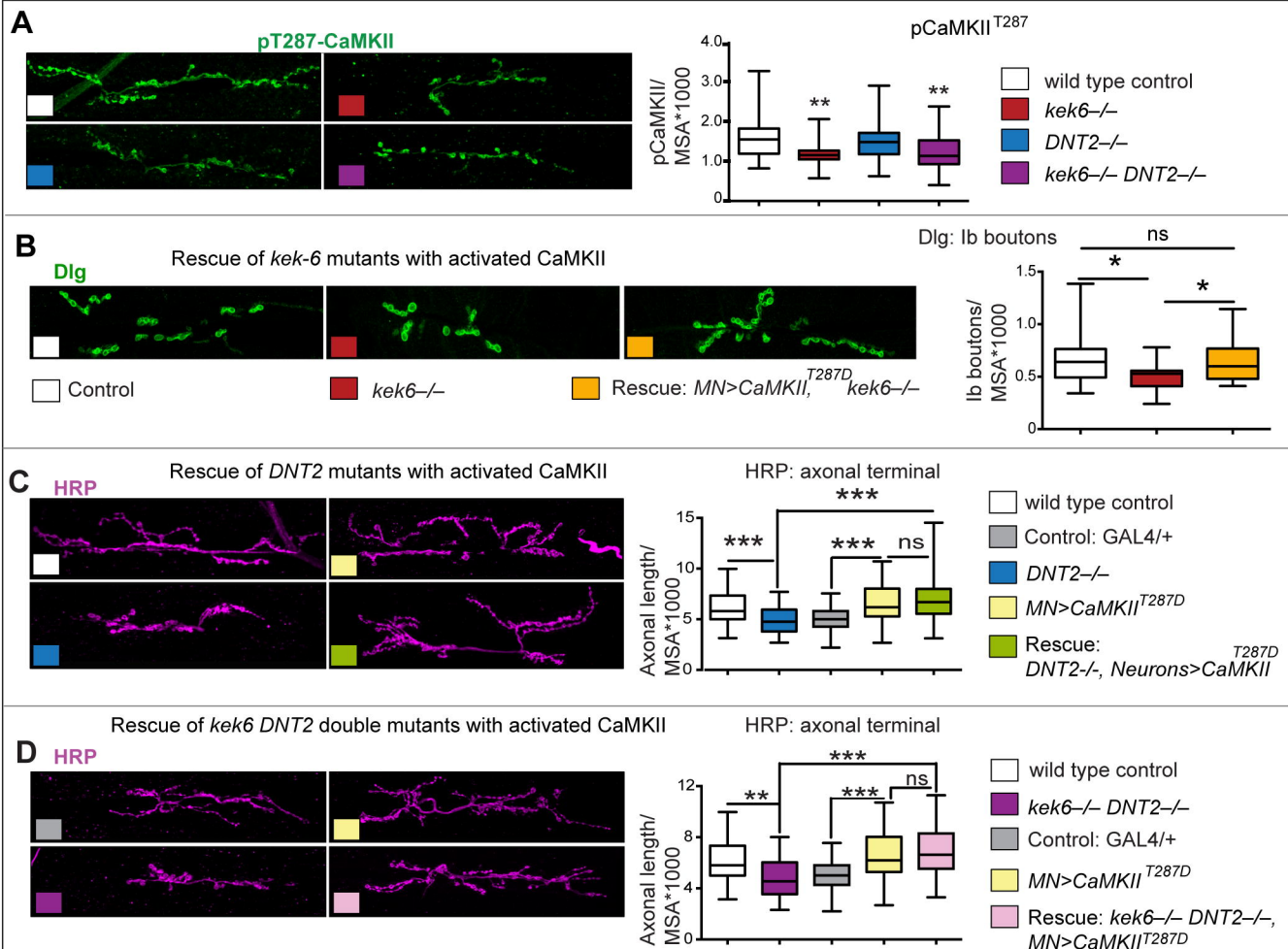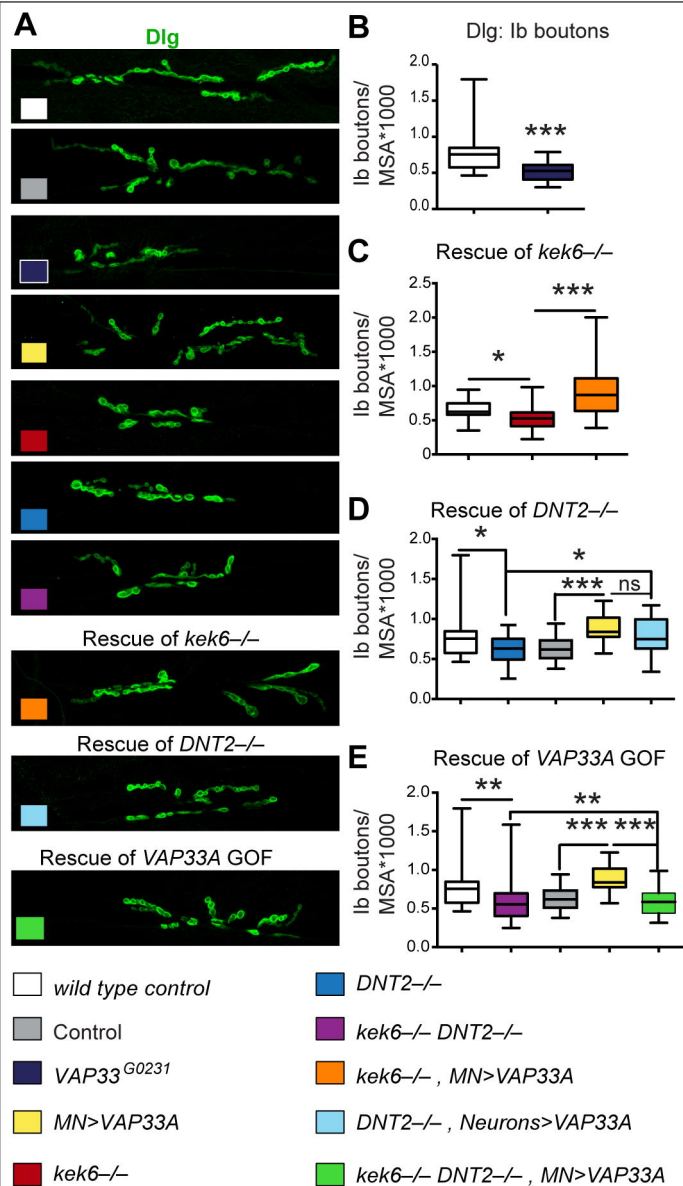Rescue: kek6–/– DNT2–/–, MN>CaMKII^{T287D}

Figure 11   VAP33A functions downstream of Kek-6

Figure 12   Retrograde DNT2 binds pre-synaptic Kek-6 activating CaMKII and regulating structural synaptic plasticity