Gαq Mediates Clozapine Effects in *Caenorhabditis elegans*

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ABSTRACT

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21   Clozapine binds and has significant effects on multiple neurotransmitter receptors, notably

22   including some dopamine receptors. Downstream of these receptors, clozapine affects the

23   balance of Gi- and Gq-dependent second-messenger signaling. We used *Caenorhabiditis elegans*

24   as a genetic model to study further how clozapine affects both dopamine receptors and

25   downstream Gq mediated signaling. Four of six worm dopamine receptor orthologs, *dop-1, dop-*

26   *2, dop-4*, and *dop-5* produced resistance to clozapine induced developmental delay when

27   mutated, suggesting that both type I and type II dopamine receptors mediate the behavioral

28   effects of clozapine in *C. elegans*. Beyond these receptors, reduction of function of one of the G

29   proteins, *egl-30* (Gαq), produced greatly increased susceptibility to clozapine. Gαq has multiple

30   known downstream effects. Among these is the control of acetylcholine release, which is in

31   balance with monoamines in the human brain and is another target of clozapine and other

32   antipsychotic drugs. We tested for downstream effects on acetylcholine at the neuromuscular

33   junction upon clozapine treatment but found no evidence for effects of clozapine. In contrast,

34   modulation of Gαq upstream leads to worms that are either more resistant or more susceptible to

35   clozapine, emphasizing the importance of Gαq proteins in mediating effects of clozapine. A

36   genetic screen for suppressors of *egl-30* recovered eight mutants. By characterizing the

37   behavioral effects of these mutants, we found that clozapine exerts its function on development

38   by affecting Gαq signaling through control of the pharyngeal pumping rate. A whole-genome

39   sequencing technique was utilized and identified a list of candidate genes for these suppressor

40   mutations. Further characterization of these mutants promises the discovery of novel components

41   participating in Gαq signaling and a better understanding of the mechanisms of action of

42   clozapine.

3

Introduction

43

44 Clozapine is an atypical antipsychotic drug (APD), mainly used for schizophrenia that does not

45 improve following the use of standard antipsychotic medications. The pharmacology of

46 clozapine shares some features with other APDs, but other features are unique, and its sites of

47 action are especially complex.

48

49 Standard antipsychotic drugs are thought to achieve some of their important effects (both

50 beneficial and unwanted) by acting as antagonists or partial agonists at D2 receptors (JONES AND

51 PILOWSKY 2002). In humans, there are five distinct G-protein coupled dopamine (DA) receptors,

52 and they are divided into two major subtypes: D1-like (D1 and D5) and D2-like (D2, D3 and D4)

53 (CUMMING 2011). These two classifications were originally assigned based on the receptor's

54 effect on cyclic AMP levels, but also reflect genetic homologies. Selective pharmacological

55 agents and receptor knockouts have been applied to demonstrate that signaling through D1-like

56 and D2-like receptors have opposite effects on many aspects of cell activation and some

57 behaviors (KELLY et $al.$ 1998; GONG et $al.$ 1999; MCNAMARA et $al.$ 2003). However,

58 simultaneous stimulation of both D1 and D2 type receptors shows that these receptors also have

59 synergistic effects on certain behaviors (PLAZNIK et $al.$ 1989; GONG et $al.$ 1999). By comparison

60 to standard antipsychotic medications, clozapine binds weakly to D2, more strongly to D4,

61 among dopamine type II receptors, and most strongly to Type I dopamine receptors, including

62 the D1 receptor. It also binds with high affinity to histamine (H1), acetylcholine muscarinic

63 (M1), α-adrenergic (α1), and multiple serotonin (5-HT$_{2A}$, 5-HT$_{2C}$, 5-HT$_7$) receptors (NAHEED

64 AND GREEN 2001).

65   The *Caenorhabditis elegans* (*C. elegans)* genome encodes six genes for dopamine receptors, and

66   four of these genes have been validated as orthologous to human DA genes, based on sequence

67   similarities, pharmacological profiles, and biochemical properties (SUO *et al.* 2002; SUO *et al.*

68   2003; CHASE *et al.* 2004; SUGIURA *et al.* 2005). As in humans, these receptors are divided into

69   two categories with opposite effects in modulating cyclic AMP levels; agonists at *dop-1* and *dop-*

70   *4* upregulate cyclic AMP levels and agonists at *dop-2* and *dop-3* do the opposite. The other two

71   orthologs, T02E9.3 and C24A8.1, named *dop-5* and *dop-6*, have similarities to the *dop-3*

72   sequence, but they have not yet been experimentally validated to be D2-like. Signaling

73   transduction mediated by these dopamine receptors controls multiple worm behaviors, including

74   locomotion, egg laying, defecation, basal motor activity, sensation/response to food sources, and

75   habituation to touch (MCDONALD *et al.* 2006). DOP-1 and DOP-3 have antagonistic effects on

76   locomotion, by acting in the same motor neurons, which co-express the receptors and which are

77   not postsynaptic to dopaminergic neurons.

78

79   Direct effects on DA and other receptors are the start of a cascade of reactions which next passes

80   through G proteins, as second messengers. A non-biased genetic screen identified two

81   antagonistic G proteins, Gαo and Gαq, activated by these receptors and mediating their

82   downstream effects (CHASE *et al.* 2004). Gα-GTP and Gβγ dimers transmit receptor-generated

83   signals to downstream effector molecules and protein-binding partners until the intrinsic GTPase

84   activity of Gα hydrolyzes GTP to GDP and the inactive subunits re-associate. G proteins are

85   localized and function primarily at the plasma membrane, in contact with cell surface receptors,

86   but also function intracellularly at endosomes (SLESSAREVA *et al.* 2006)., Gαq activates

87   phospholipase C-β (PLCβ), thereby generating the second messengers inositol-1,4,5-triphosphate

88   (IP3) and diacylglycerol (DAG). These, in turn, release stored Ca^{2+} and activate protein kinase C.

89   Gαq also activates GEF p63RhoGEF (ARHGEF25) and its homologs Trio and Duet. These

90   tertiary messengers subsequently modulate numerous enzymatic processes, metabolic pathways,

91   components of the cytoskeleton and the release of extracellular signaling molecules.

92

93   *C. elegans* has 21 Gα, 2 Gβ and 2 Gγ genes and it expresses one ortholog of each of the four

94   mammalian families GSA-1(Gs), GOA-1(Gi/o), EGL-30 (Gq) and GPA-12 (G12). The

95   remaining *C. elegans* Gα subunits do not share sufficient homology to mammalian Gα proteins

96   to allow classification (BASTIANI AND MENDEL 2006). They may play roles similar to the four

97   types of G proteins, e.g., GPA-14 is able to interact with DOP-2 and shows close similarity to

98   inhibitory G proteins that modulate anterior touch habituation and chemosensory associative

99   conditioning (PANDEY AND HARBINDER 2012; MERSHA *et al.* 2013). In a fully studied *C. elegans*

100   neural muscular junction, it has been discovered that a network of G protein signaling pathways

101   controls the release of synaptic vesicles and/or dense-core vesicles (PEREZ-MANSILLA AND

102   NURRISH 2009). The Gαq, Gα12 and Gαo pathways converge to control production and

103   destruction of the lipid-bound second messenger DAG at sites of neurotransmitter release. DAG

104   subsequently acts via at least two effectors, MUNC13 and PKC, to control the release of both

105   neurotransmitters and neuropeptides from motor neurons. The Gαs pathway converges with three

106   other heterotrimeric G-protein pathways downstream of DAG to regulate neuropeptide release

107   (BASTIANI AND MENDEL 2006).

108

109   G protein mediated effects of clozapine have been studied in cultured cells and mammals. In

110   cultured PC12 cells, clozapine downregulates the expression of tyrosine hydroxylase. This effect

111   can be abolished by treatment with N-ethylmaleimide, a reagent that decouples Gi/o protein from

112   its adjunct receptors, suggesting that clozapine modulates Gi/o protein signaling pathways

113   (TEJEDOR-REAL et al. 2005). In the rat brain, the serotonin receptor, 5-HT2A, recruits Gq

114   proteins promoting PLC stimulation, and clozapine antagonizes 5-HT-induced phosphoinositide

115   (PI) hydrolysis, probably by inhibiting Gq proteins (MANNOURY LA COUR et al. 2009). Recently,

116   it has been found that clozapine binds the mGluR2/2AR receptor heterocomplex, which also

117   alters the balance of Gi- and Gq-dependent signaling (FRIBOURG et al. 2011).

118

119   In our laboratory, we have used the well-documented clozapine-induced developmental

120   delay/lethality phenotype in C. elegans to study the mechanisms of action of clozapine. We

121   performed a genome-wide RNAi screen and identified 40 genes suppressing the effects of

122   clozapine and characterized some of these genes (SAUR et al. 2013; WANG et al. 2014; HAO et

123   al. 2016). To increase understanding of specific mechanisms underlying these findings, we now

124   take advantage of the known effects of clozapine on dopamine receptors and extend them to the

125   study of downstream effects on G protein signaling pathways. The new findings suggest that a

126   known G protein signaling downstream pathway is unaffected, but a non-biased genetic screen

127   revealed new G protein pathway components that could mediate effects of clozapine.

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7

129   **Materials and Methods**

130   **Nematode growth and worm strains**

131   Nematodes were cultured on NGM plates in a 20°C incubator (LEWIS AND FLEMING 1995). The

132   wild type N2 worm was used in these experiments as a control species. Other worm strains used

133   are listed as follows: DA1077: *egl-30(ad810);dpy-5(e61)/szT1[lon-2(e678)];+/szT1,* EAB66-71

134   and EAB73-74: suppressors of *egl-30(n686),* LX645: *dop-1(vs100),* LX702: *dop-2(vs105),*

135   LX703: *dop-3(vs106)*, LX704: *dop-2(vs105);dop-3(vs106),* LX705: *dop-1(vs100);dop-3(vs106),*

136   *LX706: dop-1(vs100);dop-2(vs105),* LX734: *dop-1(vs100);dop-2(vs105);dop-3(vs106)*,

137   MT1434: *egl-30(686)*, MT2609: *egl-30(n715n1190)*, NM1380: *egl-30(js126)*, PS2444: *dpy-*

138   *20(e1282);syIs36,* PS4263: *egl-30(md186);dpy-20(e1282);syIs105*, RB1254: *dop-4(ok1321),*

139   RB1680: *dop-6(ok2090),* RB785: *dop-5(ok568)*. The other worm strains employed can be seen in

140   Table 1.

141

142   **Developmental drug assay**

143   The developmental drug assay was performed as previously described in (HAO AND BUTTNER

144   2014). In brief, well-grown adult animals were treated in bleach solution (hypochlorite:1 N

145   NaOH:ddH2O(1:4:5)) to obtain synchronized eggs. 25 synchronized eggs were then placed onto

146   12-well NGM assay plates containing variable concentrations of drugs. The drugs were initially

147   dissolved in DMSO to obtain 80 mM stock solutions, which were diluted into 1.7 mM acetic

148   acid. Treated worms were transferred onto the NGM plates. Every 24 hours thereafter, the plates

149   were observed and the developmental status of the worms was recorded. For each worm strain,

150   the experiment was repeated at least three times. The concentration of clozapine was adjusted

151   according to the sensitivity of the tested worm strain. We present results from the third day of

152   observation, as results at this time optimally identify and represent the phenotype of strains with

153   different growth characteristics.

154

155   **Worm Behavioral assays**

156   The worms used for behavioral assays were staged young adult worms. Unless stated otherwise,

157   these were obtained by picking well-grown L4 worms onto NGM plates 24 h before the assay to

158   allow them grow to young adulthood. For the pharyngeal pumping assay, ten worms were placed

159   on NGM assay plates containing various concentrations of drugs, allowed to adapt for 30 min,

160   then, the number of contractions of the pharyngeal bulb was counted for 20 s for each worm. For

161   the aldicarb assay, the NGM plates were supplemented with 1 mM aldicarb. Thirty worms were

162   placed onto the assay plates, observed every 30 min and scored as moving or paralyzed worms.

163   Paralyzed worms did not move when poked by the worm pick. For the locomotion assay, ten

164   young adult worms were placed on NGM plates seeded with OP50 *E. coli*. After adaptation for 5

165   min, the number of wiggles of the body was counted for 30 s for each worm. For the egg

166   retention assay, well-fed young adult worms were picked onto NGM plates with food and

167   allowed to grow for 24 h. The assayed worms were picked onto a slide with an agarose pad with

168   20 mM sodium azide, and observed under a Zeiss Axio2 microscope using the 100x objective

169   lens. The number of eggs in the belly were counted. All assays were repeated three times and

170   two-tail t-tests were used to compare the mutant strain with the control strain.

171

172   **Genetic screen for suppressors of *egl-30(n686)***

9

173 Synchronized L4 worms were treated with 50 µM ethyl methanesulfonate and shaken for 4 hrs.

174 The treated worms (P0) were collected and washed twice with M9 buffer and transferred onto

175 two NGM plates to grow for 24 hrs. Ten adult worms were transferred onto each of ten fresh

176 plates and allowed to lay eggs for 4 hrs. Then they were transferred onto another ten fresh plates

177 and allowed to lay eggs (F1) overnight. After they grew to adulthood, ten F1 worms were

178 transferred onto 60 NGM plates containing 250 or 350 µM clozapine, and allowed to lay eggs

179 (F2) overnight. After 1-2 days, ten surviving F2 worms were transferred onto each of 60 NGM

180 plates and allowed to grow to adulthood. Then six F2 worms were transferred onto NGM plates

181 containing 300 µM clozapine (*egl-30(n686)* was also picked as a control) and allowed to lay 25

182 eggs (F3), after which the adult worms were removed. After 2-3 days, if there were more worms

183 surviving in a plate compared to the *egl-30* control, the plate of worms were classified as likely

184 suppressors. The suppressors were recovered and validated by testing them with a standard

185 clozapine developmental delay assay (HAO AND BUTTNER 2014).

186

Whole-genome sequencing and data analysis

188 Worms were grown on 10-20 8.5 cm NGM plates until they were gravid adults, then washed

189 with M9 buffer into a 50 ml tube and centrifuged at 2000xg for 3 min. The supernatant was

190 removed and 30 ml of bleach solution was added. Then the tube was inverted for 7-10 min until

191 all the worms were dissolved, followed by centrifugation at 1500xg for 1 min to collect eggs.

192 The eggs were washed once with M9 buffer, transferred onto 10 5.5 cm unseeded NGM plates,

193 and incubated overnight to allow the eggs to hatch. The hatched L1 worms were washed off the

194 plates with M9 buffer and collected by centrifugation at 1500xg for 1 min, then washed twice

195 with M9 buffer. The clean L1 worms were used immediately or stored at -80°C. DNA was

10

196   isolated via a Gentra Puregene Kit (Qiagen). The concentration of the final DNA preparation was

197   determined using the Qubit assay (Invitrogen). 1 µg DNA was used for preparing the sequencing

198   library according to the TruSeq®DNA PCR-free sample preparation kit (Illumina). DNA

199   sequencing was performed using an Illumina HiSeq sequencer.

200

201   The sequencing results were analyzed at the high-performance computation facility of the

202   Partners Healthcare System. The reads were mapped to the reference genome (WS220) with

203   Bowtie-0.12.7, and the variant calls were done with Samtools. The variants were checked in igv.

204   We only focused on mutations that occurred in coding regions and splicing sites at which

205   variants could potentially cause disturbance of protein structures.

206

207   Strains are available upon request.

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Results

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208

209 **Dopamine receptors mediate effects of clozapine**

210 There are six dopamine receptor orthologs in the *C. elegans* genome, and mutants in each

211 ortholog are available. We performed developmental assays by growing the mutants and wild

212 type worms in plates treated with 0, 300, 400, and 500 µM clozapine. As shown in Fig. 1A, the

213 lethality rate of wild type worms was high and surviving worms were at the L1 stage. By

214 comparison, the lethality rates of *dop-1, dop-2, dop-2;dop-3, dop-1;dop-3, dop-1:dop-2, dop-*

215 *1;dop-2;dop-3* mutants were much lower and some of the worms developed to the L2 stage. By

216 comparison, the lethality rate of *dop-3* mutants was as high as that of the wild type strain. These

217 observations, that *dop-1* and *dop-2* mutants were resistant to clozapine, while *dop-3* mutants

218 were not resistant to clozapine, suggest that both *dop-1* and *dop-2* mediate effects of clozapine,

219 but *dop-3* does not. We also tested mutants of the other three dopamine receptors, *dop-4, dop-5*

220 and *dop-6*, and found that *dop-4* and *dop-5* mutants were resistant to the effects of clozapine, but

221 *dop-6* mutants were not (Fig. 1B).

222

223 **Gαq proteins mediate effects of clozapine**

224 Dopamine receptors are G protein coupled, and the alpha subunit of G protein has been found to

225 be associated with the effects of clozapine in mammalian models. To test for this association in

226 *C. elegans*, we studied mutants of the Gαq ortholog, *egl-30*, in worms. Compared to wild type,

227 the lethality rate of a reduction of function (*rf*) mutant, *egl-30(n686),* was dramatically increased

228 and development was delayed. Conversely, the lethality rate of a constitutively active mutant,

229 *egl-30(js126)*, was slightly reduced and development was promoted (Fig. 2A). To confirm this

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230   observation, we conducted drug assays on two other *rf* mutant alleles, *egl-30(ad810)* and *egl-*

231   *30(n715n1190),* and found that they were also susceptible to clozapine (Fig. 2B). We also tested

232   two worm strains carrying the integrated transgene of *egl-30* DNA sequence in either wild type

233   background or an *egl-30* mutation background, supposed to have higher Gαq level than wild

234   type, and found that they both reduced lethality rates and promoted development, compared to

235   wild type (Fig. 2B).

236

237   **Various known downstream components of the Gαq signaling transduction pathway are**

238   **not involved in mediating effects of clozapine**

239   The *egl-30* gene is expressed ubiquitously and required for worm viability, locomotion, egg

240   laying, synaptic transmission and pharyngeal pumping. We used egg laying and locomotion

241   phenotypes to study downstream behavioral effects of changes in Gq signaling pathways. We

242   also studied one pathway related to synaptic transmission. Gα protein-involved signaling

243   controls the release of acetylcholine and other neuropeptides (Fig. 3).

244

245   We performed developmental drug assays on three types of Gα proteins, *goa-1* (Gαo), *gpa-12*

246   (Gα12), *gsa-1* (Gαs) and found that their mutants are no different than wild type in response to

247   clozapine (Fig. 3 and Table 1).

248

249   In the Gq and Gαs signaling pathways, we conducted developmental drug assays on the

250   downstream components of the Gq signaling pathway, namely, *egl-8, unc-73, dgk-1, unc-13,*

13

251   *pkc-1*, and found that *dgk-1* suppressed the effects of clozapine, while *egl-8* was similar to wild

252   type or slightly suppressed the effects of clozapine. By comparison, *unc-73, unc-13*, and *pkc-1*

253   strains all responded to clozapine the same as wild type strains (Table 1). In addition,

254   developmental drug assays performed on the downstream components of the Gαs signaling

255   pathway, *acy-1, pde-4*, and *kin-2,* showed that they all responded to clozapine the same as wild

256   type strains (Table 1). These observations suggested that these known downstream components

257   of Gq and other Gα signaling pathways were not involved in mediating the effects of clozapine.

258

Gαq modulators alter effects of clozapine

260   We investigated the role of modulators of Gαq by conducting developmental assays and

261   discovered that mutants of *ric-8*, a non-receptor activator of Gαq in both *C. elegans* and

262   mammals, are resistant to clozapine (Fig. 3, Table 1). Mutants of three other modulators of Gαq,

263   *cnb-1, eat-16*, and *rsbp-1* were found to be more susceptible to clozapine (Table 1).

264

Isolation and characterization of Gαq mutant suppressors of clozapine effects

266   We performed a forward genetic screen for suppressors of *egl-30(n686)*, taking advantage of the

267   hypersensitivity of *egl-30(n686)* to clozapine. We found eight mutants that were more resistant

268   to clozapine than *egl-30(n686)*, and these suppressors were named EAB66-71 and EAB73-74.

269   The strongest suppressor was EAB66, which was even more resistant to clozapine than wild type

270   (data not shown). We characterized these mutants for other behavioral phenotypes and compared

271   them to wild type and *egl-30(n686)* worms. Aldicarb is an inhibitor of cholinesterase, causing

272   rapid accumulation of acetylcholine at the synaptic cleft, and thereby paralysis of the worms. The

14

273   *egl-30(n686)* mutant is resistant to aldicarb compared to wild type. The suppressor EAB66 is

274   phenotypically like the wild type worm, whereas EAB67 remains phenotypically like the *egl-*

275   *30(n686)* worm (Fig. 4A). In the locomotion assay, strains carrying the suppressor EAB66 move

276   faster than *egl-30(n686)*, but they do not move at the wild type level; whereas the EAB67 strain

277   is even slower than *egl-30(n686)* (Fig. 4B). As for the egg retention assay, EAB66 and EAB67

278   strains are both more similar to *egl-30(n686)* strains than wild type worms (Fig. 4C). However,

279   in the pharyngeal pumping assay, all the suppressors show activity that is statistically

280   significantly different from *egl-30(n686)*, except EAB73 at 450 µM (Fig. 4D).

281

Candidate genes for Gαq suppressors revealed by whole-genome sequencing

283   To identify the specific genes coding for the suppressor mutants, we performed whole-genome

284   sequencing of these mutants and compared the sequences of wild type and *egl-30(n686)* worms.

285   As shown in Table 2, there are two to five mutations occurring in these mutants, each of which

286   changes protein coding sequences. Most of the mutations introduce a stop codon in a coding

287   region; but there are four mutations that change splicing sites; and one mutation changes the

288   initial start codon (M/V) in EAB68 (Table 2).

Discussion

289

290   In the worm model, dopamine receptors participate in mediating the effects of clozapine in

291   producing developmental delay/lethality. Interestingly, when mutated, four out of six dopamine

292   receptors, including both type I and type II dopamine receptors, relieve clozapine-induced

293   developmental phenotypes (Fig. 1). These findings cannot be explained by the simple model in

294   which the two types of dopamine receptors have purely opposite and competing effects at the

295   level of production of cAMP, through activation of Gαs and Gαo. Our observation that neither

296   Gαs nor Gαo mutants display behavioral differences from wild type strains in drug assays is also

297   consistent with a more complex interaction of dopamine receptors and G proteins (Table 1).

298   Notably, there is growing evidence that G protein coupled receptors (GPCRs) execute their

299   functions through oligomerization, including homomerization and heteromerization (FERRE AND

300   FRANCO 2010). Oligomerization allows receptors to achieve new properties, including changes

301   in ligand recognition, G protein-coupling and trafficking (FERRE AND FRANCO 2010). For

302   example, while the D1-D3 receptor heteromer couples to Gs (FIORENTINI *et al.* 2010), the D1-D2

303   and D2-D5 heteromers couple preferentially to Gq proteins (HASBI *et al.* 2010). Clozapine may

304   exert its effects on development through interactions with and modulation of such oligomers of

305   dopamine receptors.

306

307   Based on our genetic studies on the components in the four G protein-involved signaling

308   networks (Fig. 2, Table 1), only Gq and its modulators appear to strongly mediate the effects of

309   clozapine. The classical model that Gs and Go balance cAMP production and metabolism is not

310   supported by our findings, since the mutants of Gs and its downstream components do not differ

311   from wild type in their drug response. Gq and Go may form a signaling balance as suggested by

16

312   Fribourg et al. (FRIBOURG *et al.* 2011). A Go/Gq heterotrimeric G protein signaling network

313   expressed throughout the nervous system has been shown to regulate locomotion rate, partly by

314   affecting serotonergic neurotransmission. And loss-of-function (*lf*) mutations in *goa-1* (Gαo)

315   cause hyperactivity, whereas *lf* mutations in *egl-30* (Gαq) cause severe lethargy (MILLER *et al.*

316   1999; NURRISH *et al.* 1999). However, while *egl-30* Gαq mutants are highly susceptible to

317   clozapine (Fig. 2), the *goa-1* (Gαo) mutant does not display a resistant phenotype upon clozapine

318   treatment (Table 1). Furthermore, the downstream components, i.e., in the PLC signaling

319   pathway that facilitates synaptic transmission by body-wall muscle motor neurons, do not appear

320   to mediate clozapine effects, as their mutants have the same response as wild type strains (Table

321   1). Taken together, these observations indicate that the effects of clozapine on worm

322   development are not mediated by these known Gαq downstream signaling elements.

323

324   To explore how Gq proteins mediate the effects of clozapine, we performed a genetic screen and

325   identified eight suppressing mutants that were more resistant to the behavioral effects of

326   clozapine than the *rf* of *egl-30* strain. Since the *rf* of *egl-30* exhibited phenotypes including

327   decreased pharyngeal pumping and locomotion rates, reduced sensitivity to aldicarb, and high

328   retention of eggs, we tested whether these behaviors were affected by the suppressing mutations.

329   We tested two suppressors, EAB66 and EAB67, and found that aldicarb sensitivity, locomotion

330   rate and egg retention were changed at baseline in ways similar to *egl*-30, but EAB66 and

331   EAB67 did not have the characteristics of *egl*-30 in altering response to clozapine (Fig. 4). The

332   genes affected by these suppressors might not be involved in the developmental/lethality

333   phenotypes induced by clozapine. In contrast, the pharyngeal pumping rates of all the

334   suppressors recovered to the wild type levels (Fig. 4), indicating that this behavior does

17

335 correspond to the effects of clozapine on development, which is in agreement with our

336 previously reported findings (HAO *et al.* 2016).

337

338 Whole-genome sequencing revealed that each mutant strain carried 2-5 mutations that would

339 likely change protein structures. Most of the candidate genes in these suppressors are novel.

340 Further studies may identify the components that mediate the effects of clozapine through the Gq

341 protein.

342

343 In summary, we studied the actions of clozapine, using *C. elegans* as a genetic model, and

344 observed that both type I and type II dopamine receptors mediate the behavioral effects of

345 clozapine. Further, the results suggest that dopamine receptors form oligomers to signal through

346 Gq proteins. We also found that clozapine exerts its function on development by modulating Gq

347 signaling, which controls the pharyngeal pumping rate. The mutants we identified can be studied

348 further and those studies promise more interesting discoveries involving Gq signaling and a

349 better understanding of the mechanism of action and effects of clozapine beyond its direct effects

350 on cell surface receptors.

Acknowledgements

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434

435   **Figure Legends**

436   **Fig. 1. Dopamine receptors mediate clozapine effects. (A)** Dopamine receptor mutantss, *dop-*

437   *1(vs100), dop-2(vs105), dop-3(vs106)*, and combinations of these mutants, *dop-2(vs105);dop-*

438   *3(vs106), dop-1(vs100);dop-3(vs106), dop-2(vs105);dop-3(vs106), dop-1(vs100);dop-*

439   *2(vs105);dop-3(vs106)* were treated with clozapine at 0, 300, 400, 500 µM. **(B)** Dopamine

440   receptor mutants, *dop-4(ok1321), dop-5(ok568), and dop-6(ok2090)* were treated with clozapine

441   at 0, 300, 400, 500 µM.

442

443   **Fig. 2. Gαq mediates clozapine effects.** (A) Two Gαq mutants, one reduction of function (*rf*)

444   mutant *egl-30(686)* and one constitutive activity mutant *egl-30(js126)*, was compared with that

445   of the wild-type N2 strain by testing them in a series of concentrations of clozapine at 0, 50, 100,

446   1*50, 200, 250, 300* and *350* µM. (B) Two other *egl-30(rf)* mutants, *ad810, n715n1190*, and two

447   worm strains carrying the over-expressed genomic rescue constructs, *dpy-20(e1282);egl-*

448   *30*genomic and *egl-30(md186);dpy-20(e1282);gfp/egl-30*genomic were treated with clozapine at

449   0, 300, 400, 500 µM.

450

451   **Fig. 3. Testing downstream clozapine effects via an established Gα signaling pathway that**

452   **regulates Acetylcholine receptor and neuropeptide release in *C. elegans*.** Adapted from

453   Perez-Mansilla and Nurrish (2009). The green colored components indicate mutants that are

454   resistant to clozapine; the red colored components indicate mutants that are susceptible to

455   clozapine; and the orange colored components indicate mutants that are phenotypically similar to

456   wild type strains. Mutants in the black components were not tested.

457

458 **Fig. 4. Characterization of the *egl-30* suppressors.** (A) N2, MT1434 and two EAB66-67

459 suppressor strains were treated with 1 μM aldicarb and examined for movement at 0, 0.5, 1, 1.5,

460 2, 2.5, 3, 3.5, 4, 4.5 hrs. The data are from triplicated experiments with total numbers of tested

461 worms N=70. (B) Locomotion assays of the N2, MT1434 and EAB66-67 suppressor strains.

462 There are statistically significant differences in phenotype among these four strains, P<0.001.

463 The data are from triplicated experiments with a total number of tested worms N=35. (C) Egg

464 retention assays of the wild type N2, MT1434 and EAB66-67 strains. Comparison of these four

465 strains again shows statistically significant differences, P<0.05. The data are from triplicated

466 experiments with a total number of tested worms N=60. (D) Pharyngeal pumping assays of wild

467 type (grey bar), MT1434 (black bar) and eight MT1434 suppressor strains (EAB66-71, 73, 74)

468 (color bars). Comparisons between each of the suppressors and the MT1434 strain show

469 statistically significant differences, but differences are not observed between the suppressor

470 bearing strains and wild type strains. (* indicates p<0.001 between each of the mutants and

471 MT1434.) The data are from triplicated experiments with a total number of tested worms N=30.

472

473 **Table 1. Summary of drug assays on worm strains in known *C. elegans* Gα signaling**

474 **transduction.** +: Weak suppressor, ++: Intermediate suppressor, +++: Strong suppressor, –:

475 Weak enhancer, ––: Intermediate enhancer, –––: Strong enhancer, ±: Wild-type.

Ortholog in human	Gene	Allele	Strain	Developmental assays				Concluded phenotype
Gα₀	*goa-1*	*sa734*	DG1856	±	±	- -		**WT**
PLC β	*egl-8*	*n488*	MT1083	++	+	±		**Suppressor**
HMUNC13	*unc-13*	*e51*	MT7929	+	- -	±		**WT**
DGKQ	*dgk-1*	*sy428*	PS2627	++	+++	+		**Suppressor**
PRKCE	*pkc-1*	*ok563*	RB781	- -	- -	++	+	**WT**
GNEF	*unc-73*	*e936*	CB936	+	±	±		**WT**
RGS6,7,9,11	*eat-16*	*sa609*	JT609	±	- - -	- - -	-	**Enhancer**
Calcineurin B	*cnb-1*	*jh103*	KJ300	- -	- -	- -	- -	**Enhancer**
None	*rsbp-1*	*vs163*	LX1270	- - -	- -	- - -		**Enhancer**
Synembryn-A	*ric-8*	*md303*	RM1702	++	+++	++		**Suppressor**
GNA12 and 13	*gpa-12*	*pk322*	NL594	±	±	±		**WT**
GNAL and S	*gsa-1*	*ce81*	KG421	±	±	±		**WT**
ADCY9	*acy-1*	*ce2*	KG518	±	±	±		**WT**
PDE4A-D	*pde-4*	*ce268*	KG744	±	±	±		**WT**
PRKAR1A-B, CNBD1	*kin-2*	*ce179*	KG532	±	±	±		**WT**

476

477 **Table 2. Candidate mutated genes of *egl-30(n686)* suppressors revealed by whole-genome**

478 **sequencing.**

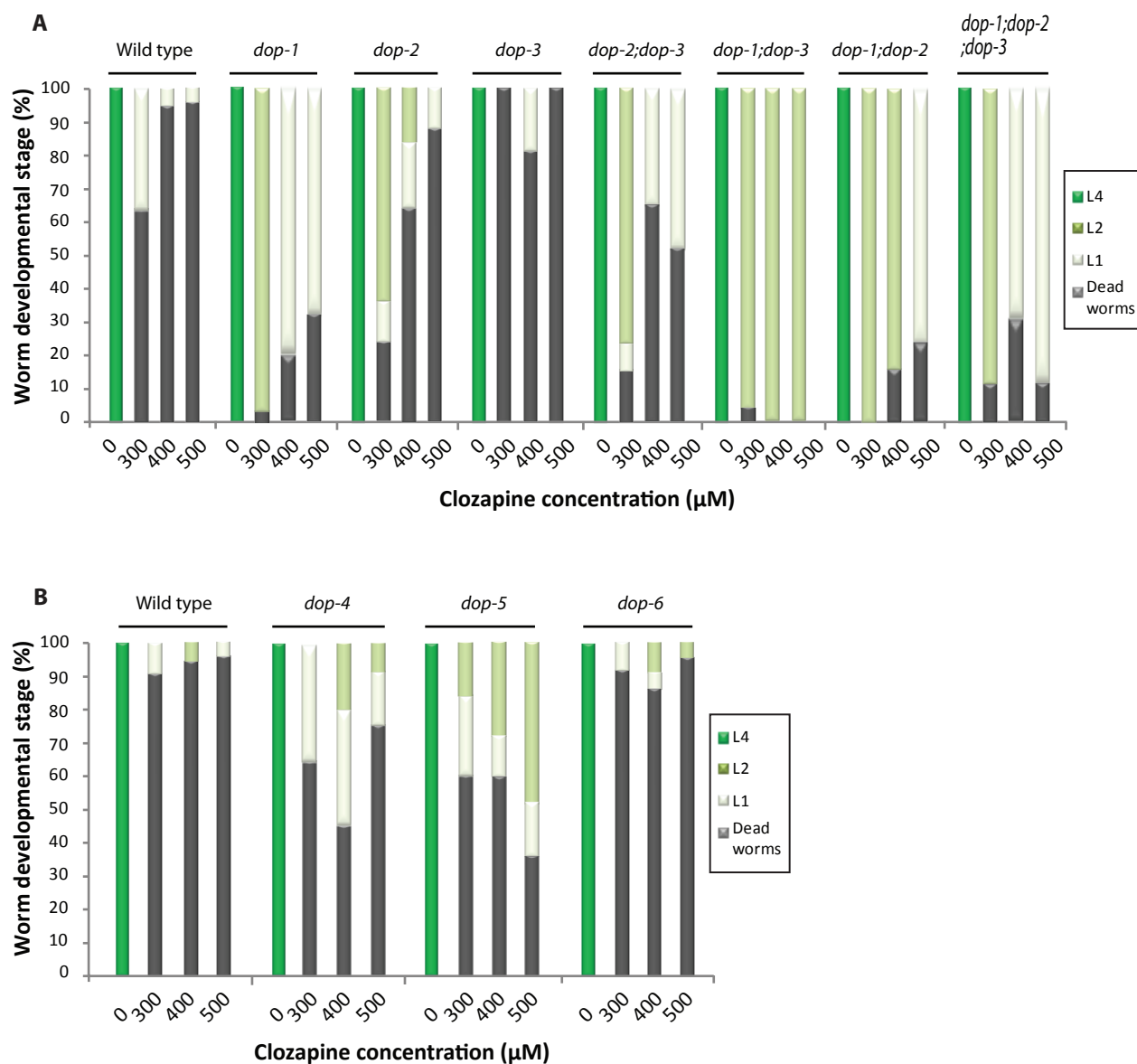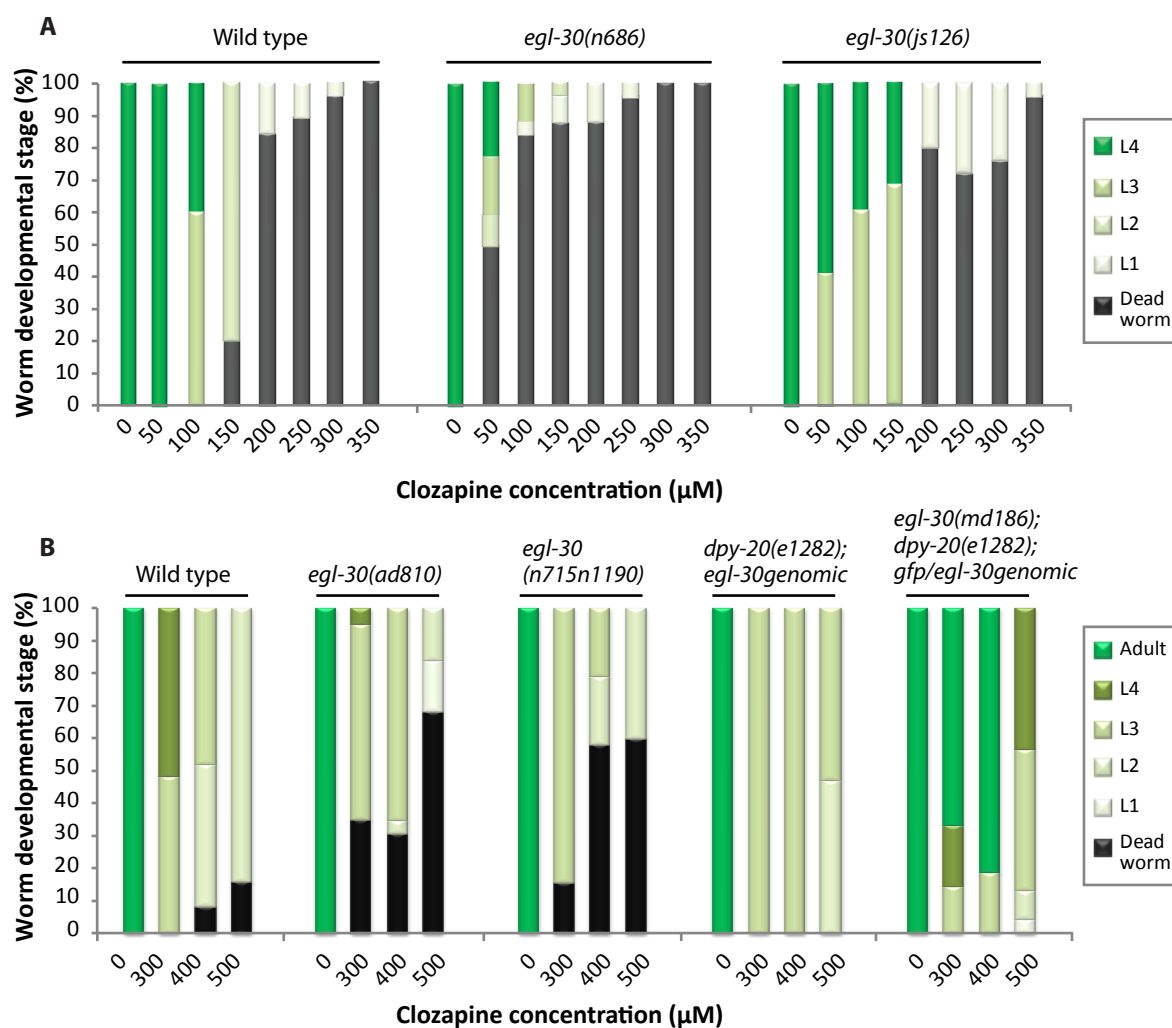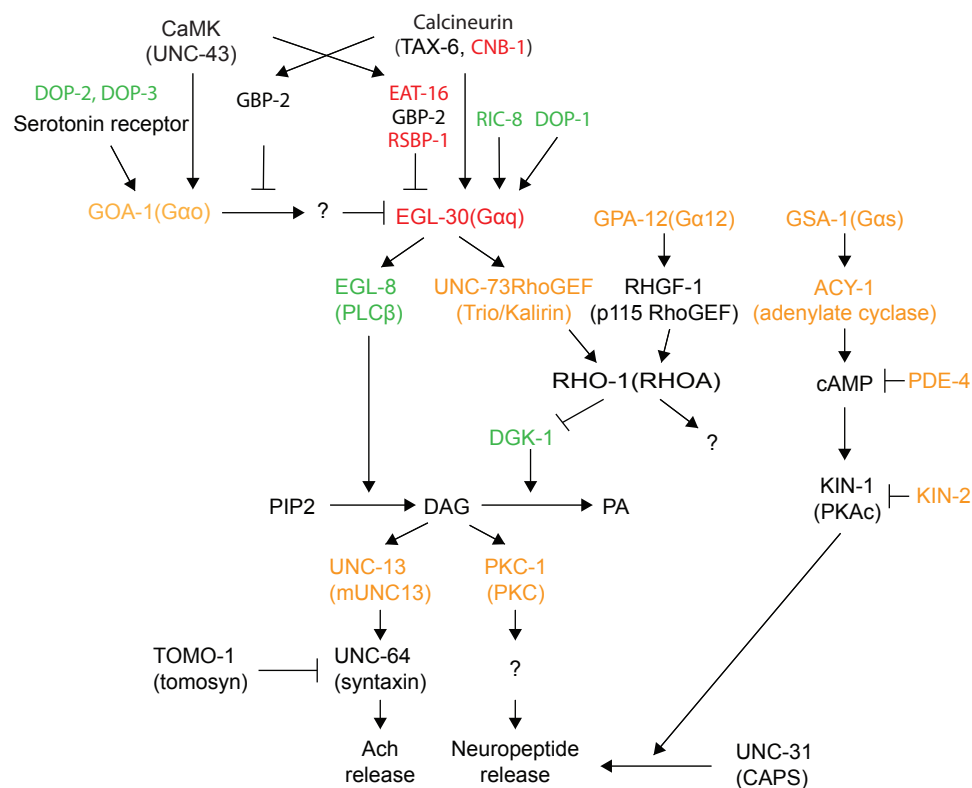| Suppressor	Location	Gene name or putative function	Site	nn change	AA change
EAB66	F08G5.7 /IV	*clec-184*	12433358	C/T	Q/Stop
	M02b1.1/IV	*srf-3*	12850684	C/T	Q/Stop
	C31B8.7/V	No info, only in nematodes	2901829	C/T	Splicing site
	T08A9.1/X	*atg-11*	7339350	C/T	Q/Stop
EAB67	T13B5.7/II	*lips-13*	1115919	G/A	Q/Stop
	C41A3.1/V	*fasn-1* (fatty acid synthase)	5430972	C/T	Q/Stop
EAB68	Y39H10A.4/V	Not known function; Only in nematode	3733499	C/T	Splicing site
	srj-27/V	Predicted to have transmembrane signaling receptor activity, based on protein domain information. Only exists in nematode	15126479	G/A	Q/Stop
	zc13.10/X	Not known function; Only in nematode	902597	T/C	Initial M/V
	F21G4.3/X	Not known function; Only in nematode	9896512	C/T	R/Stop
	zc506.1/X	Ortholog of human EDEM3 (ER degradation enhancer); predicted to have mannosyl-oligosaccharide 1,2-alpha-mannosidase activity and calcium ion binding activity, based on protein domain information.	9963398	C/T	Q/Stop
EAB69	*clec-131*/I	C-type lectin, only existed in nematode.	3575866	C/T	W/Stop
	rmd-3/I	Ortholog of human RMDN2 and RMDN3 (regulator of microtubule dynamics).	11339397	C/T	Splicing site
EAB70	Y87G2A.7/I	*nyn-2*, involved in RNAi	13574346	C/T	Q/Stop
	Y66D12A.14/III	Aminoadipate-semialdehyde dehydrogenase	11544129	C/T	Q/Stop
EAB71	ZK1055.7/V	Involved in innate immune response	6603050	G/A	Q/Stop
	C41A3.1/X	Ortholog of human FASN (fatty acid synthase).	5430972	C/T	Q/Stop
EAB73	*unc-89*	Encodes several protein isoforms variably characterized by the presence or absence of SH3, DH, PH, immunoglobulin and myosin light chain kinase (MLCK)-like protein kinase domains	4082080	A/T	R/Stop
	Y54B9A.1	Kinesin-like protein	8078419	G/A	W/Stop
EAB74	*srv-17*	7TM GPCR, serpentine receptor class v (Srv)	5869831	C/T	Splicing site
	F23D12.2	Encodes a protein containing an F-box and FTH/DUF38 motifs, mediating protein-protein interaction.	14416566	G/A	R/Stop

479

Figure 1

Figure 2

Figure 3

Figure 4