Rab-3 and unc-18 Interactions in Alcohol Sensitivity Are Distinct from Synaptic Transmission

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Abstract

The molecular mechanisms underlying sensitivity to alcohol are incompletely understood. Recent research has highlighted the involvement of two presynaptic proteins, Munc18 and Rab3. We have previously characterised biochemically a number of specific Munc18 point mutations including an E466K mutation that augments a direct Rab3 interaction. Here the phenotypes of this and other Munc18 mutations were assessed in alcohol sensitivity and exocytosis using Caenorhabditis elegans. We found that expressing the orthologous E466K mutation (unc-18 E465K) enhanced alcohol sensitivity. This enhancement in sensitivity was surprisingly independent of rab-3. In contrast unc-18 R39C, which decreases syntaxin binding, enhanced sensitivity to alcohol in a manner requiring rab-3. Finally, overexpression of R39C could suppress partially the reduction in neurotransmitter release in rab-3 mutant worms, whereas wild-type or E465K mutants showed no rescue. These data indicate that the epistatic interactions between unc-18 and rab-3 in modulating sensitivity to alcohol are distinct from interactions affecting neurotransmitter release.

Introduction

Drug addiction is one of the leading causes of preventable death, generating a considerable financial burden to society. Indeed alcohol use and abuse can lead to increased incidence of liver disease, cardiovascular disease, cancer and other debilitating illnesses [1]. Although the environment can influence addiction, current estimates of genetic heritability range between 40-80% [2]. One significant contributing component to the genetic determination of addiction is the individual’s initial level of response as highlighted by a consistent association of alcohol addiction with polymorphisms in genes involved in alcohol metabolism [3,4]. Despite a ubiquitous prevalence in modern society, the precise physiological mechanisms of intoxication and addiction remain poorly understood. A complete understanding of the contributing factors that underlie alcohol sensitivity is therefore of potential therapeutic importance.

Current models of alcohol action within the nervous system predict low-affinity interactions of alcohol with specific target proteins or protein complexes [5]. Genetic studies of alcohol sensitivity have contra-indicated many potential targets both pre- and post-synaptic in origin [6,7]. The model organism Caenorhabditis elegans is an excellent platform for the genetic dissection of alcohol sensitivity as it has a similar dose-dependent response to exogenous alcohol to mammals [8]. Recent research from C. elegans has determined a role in alcohol sensitivity for proteins central to the exocytotic machinery, yet distinct from synaptic transmission efficacy. Loss-of-function (lof) mutations in the GTPase rab-3 reduces sensitivity to alcohol in C. elegans [9]. Similarly, a single point mutation in the protein Munc18 that inhibits SNARE complex binding specifically also reduces sensitivity to alcohol in C. elegans [10]. Both mutants also affect voluntary alcohol consumption in mice [9,11] emphasising the conservation of genetic determination of alcohol sensitivity from nematodes to mammals.

Munc18 is an essential protein in presynaptic vesicle exocytosis whose precise function remains somewhat enigmatic [12,13]. Biochemically, Munc18 binds the t-SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) syntaxin in two different modes of interaction as well as the assembled SNARE complex [14-16]. In worms, null unc-18 (e81) alleles display strong behavioural phenotypes including paralysed locomotion and resistance to inhibitors of acetylcholinesterase [17], Rab3 is a GTPase that also functions...
in exocytosis by recruiting and tethering synaptic vesicles to
the plasma membrane [18], although roles for Rab3 in late
stages of docking [19] and vesicle fusion [20] have also been
demonstrated. In worms, lof rab-3 mutants exhibit loopy, mildly
slower locomotion and are also resistant to inhibitors of
acetylcholinesterase [19].

We have previously investigated a number of point mutations
of mammalian Munc18 that alter protein interactions [21],
including an E466K gain-of-function (gof) mutation affecting
direct binding to Rab3 [22]. In this study we have investigated
the functional effects of some of these point mutations in
unc-18, the nematode orthologue of Munc18-1, in both a wild-
type and lof rab-3 genetic background. A mutation that
interferes with closed-conformation syntaxin binding (unc-18
R39C) was hypersensitive to alcohol as was the orthologous
mutation that enhances the Munc18-Rab3 interaction (unc-18
E465K). In addition overexpression of the R39C mutation
partially compensated for lof rab-3 in neurotransmitter release;
yet, was recessive to lof rab-3 in alcohol sensitivity. Conver-
sely, the E465K mutation was dominant to lof rab-3 in
alcohol sensitivity, but recessive in neurotransmitter release.
We conclude that the specific interactions between unc-18 and
rab-3 that govern exocytosis are functionally distinct from
sensitivity to alcohol.

Results

Alcohol sensitivity phenotypes of single point
mutations in unc-18

We recently demonstrated that a single point mutation
(D216N) in Munc18 acts biochemically by reducing binding to
the assembled SNARE complex and that the orthologous
mutation in C. elegans unc-18 (D214N) reduces sensitivity to
both low and high concentrations of exogenous ethanol [10].
Previously, we have biochemically characterised other point
mutations in Munc18 that affect binding to other proteins
including R39C (inhibits binding to closed-conformation
syntaxin) [23,24], P240S (inhibits binding to Mint proteins) [21]
and E466K (enhances binding to Rab3) [22]. To assess
whether these other Munc18 interactions could also affect
alcohol sensitivity we generated transgenic worms expressing
the orthologous mutations of unc-18 in a null (unc-18 e81)
background (Figure 1A) and assessed their sensitivity to
alcohol in comparison with transgenic worms expressing wild-
type unc-18. Despite a strong reduction in alcohol sensitivity,
worms that express the unc-18 D214N mutation have relatively
normal, but statistically elevated locomotion rates [10].
Similarly, the unc-18 R39C, P240S and E465K expressing
mutants exhibited qualitatively normal locomotion in
comparison to unc-18 wild-type (Table 1) although the R39C
mutants had a significant reduction in thrashing of 23% in
comparison to wild-type (Kruskal-Wallis one-way analysis of
variance on ranks with post-hoc comparison; P<0.05; N = 77
(Wt), 55 (R39C), 48 (P240S) and 55 (E465K)).

Exposing worms to high external ethanol concentrations (400
mM) causes a depression in locomotion [8,25]. In addition,
exposure of worms to low external concentrations (21 mM)
stimulates locomotion [10]. Due to the low permeability of

Figure 1. Expression of unc-18 R39C or E465K point
mutations enhances acute alcohol sensitivity. (A) Location
of the three investigated point mutations of unc-18 (R39C,
E465K and P240S) in a predicted model of UNC-18 structure.
Null unc-18 (e81) worms were rescued transgenically with
either wild-type (Wt) unc-18 or unc-18 with the indicated point
mutations. In comparison to Wt unc-18, both the R39C and
E465K point mutations increased acute sensitivity to ethanol at
(B) stimulatory levels (21 mM) and (C) depressive levels (400
mM) of external ethanol. For (A) significance was assessed by
one-way analysis of variance with post-hoc comparisons; *P<0.05; N = 28 (Wt), 20 (R39C), 22 (P240S) and 20 (E465K).
For (B) significance was assessed by Kruskal-Wallis one-way
analysis of variance with post-hoc comparisons; *P<0.05; N =
29 (Wt), 40 (R39C), 20 (P240S), 18 (E465K).

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The sensitivity of the double mutant was not significantly greater than the single mutants. Therefore, the effects of either point mutation were not additive with respect to alcohol sensitivity.

### Alcohol sensitivity phenotype of a double mutation in **unc-18**

Munc18 functions at the synapse at multiple steps in the exocytic pathway through interactions with many proteins [12,13]. We were interested to determine whether the enhanced sensitivity to alcohol of the R39C or E465K mutations had effects on sensitivity to exogenous ethanol at either the stimulatory or depressive concentrations. In contrast to the previously characterised D214N mutation, the R39C and E465K mutations enhanced sensitivity to alcohol at both the stimulatory and the depressive concentrations (Figure 1B, C). There were no effects of the P240S mutation at either concentration of ethanol. This lack of effect was perhaps unsurprising as the P240S mutation reduces binding to the Mint proteins [21] and the C. elegans orthologue of Mint, in-10, lacks the Munc18 binding domain. Therefore, both the R39C and E465K mutations of unc-18 increased sensitivity to alcohol.

### Exocytotic phenotypes of point mutations in **unc-18**

Movement of nematodes is determined by defined neural circuits, integrating sensory information to generate locomotion, as well as the strength of neuromuscular transmission. Munc18 has primarily been described as a protein essential for exocytosis [12,13]. Mice null for Munc18 have defects in both vesicle docking [26] and secretion [27]. In addition specific mutations of Munc18 can affect the kinetics of membrane fusion [15,28,29]. In C. elegans, unc-18 null mutants are paralysed and have defects in docking and neuromuscular transmission [17,30]. With respect to exocytosis, the R39C mutation causes an increase in EJP amplitude in *Drosophila* [31] and alters the kinetics of vesicle fusion in chromaffin cells [24], whereas it appears to have very little effect when expressed in *C. elegans* [23,32]. The E466K mutation enhances dense-core granule recruitment in chromaffin cells [22] and has a very mild hypersensitivity to aldicarb in *C. elegans* [33].

We next determined whether any of the mutations in unc-18 that affected alcohol sensitivity also affected the strength of synaptic transmission using the well established aldicarb sensitivity assay [34]. In this assay, quantitative changes in the rate at which a population of worms paralyse are indirect measurements of changes in synaptic strength. In comparison to worms expressing wild-type unc-18, the E465K mutants were mildly, but insignificantly, hypersensitive to aldicarb (Figure 3). In contrast, R39C worms had a small, but consistent resistance to aldicarb indicative of a reduction in signalling strength at the neuromuscular junction. We also tested the R39C/E465K double mutant in the aldicarb assay and found that the R39C mutation was dominant over E465K for the aldicarb sensitivity phenotype (Figure 3). As the two unc-18 mutations produced equivalent effects in ethanol but contrasting effects in aldicarb, we conclude that the function of the individual mutations in sensitivity to alcohol are uncorrelated with effects on synaptic transmission strength.

### Alcohol sensitivity phenotypes of point mutations in **lof rab-3**

Rab3 is a GTPase involved in the trafficking of synaptic vesicles and various aspects of exocytosis [18]. *Lof rab-3* worms are resistant to the effects of depressive concentrations of exogenous alcohol [9]. The E466K mutation of Munc18 enhances dense-core granule recruitment in chromaffin cells [22] and has a very mild hypersensitivity to aldicarb in *C. elegans* [33].

We therefore investigated whether the effects of any of our unc-18 mutations were epistatic to rab-3 by expressing in a *lof rab-3* genetic background and assaying for alcohol sensitivity. We have previously investigated the effects of specific unc-18 point mutations in both a wild-type (N2) or null (unc-18) genetic background and found similar phenotypic effects either in the presence or absence of endogenous unc-18 [35]. Similar to that seen in the null unc-18 (e817) allele, expression of R39C in *lof rab-3* (y250) caused a significant decrease in basal locomotor rate in comparison to expression of wild-type unc-18 (Table 1; one-way analysis of variance with post-hoc comparison: P<0.05; N = 25 (rab-3 y250), 25 (WT), 30 (R39C) and 30 (E465K)). The enhancement in locomotor rate by the
R39C/E465K double mutant in comparison to the single R39C mutation was also apparent in the lof rab-3 background (one-way analysis of variance with post-hoc comparison; $P<0.05$; $N = 30$ (R39C), 30 (E465K) and 30 (R39C/E465K)).

In response to low levels of alcohol, lof rab-3 worms exhibited a normal stimulation of locomotion (Figure 4A). The enhancement in alcohol-dependent stimulation by either single (R39C or E465K) or double (R39C/E465K) mutations of unc-18, however, was negated when these mutations were expressed in the lof ras-3 mutant background. As previously described [9], at depressive concentrations of ethanol lof ras-3 (y250) worms were less sensitive than Bristol N2 wild-types

**Figure 2.** The phenotypic effects of *unc-18* R39C and E465K in alcohol sensitivity are not additive. Null *unc-18* (e81) worms were rescued transgenically with wild-type (Wt) *unc-18* or *unc-18* with the indicated point mutations. In comparison to Wt *unc-18*, all three mutants had an increase in acute sensitivity to ethanol at (A) stimulatory levels (21 mM) and (B) depressive levels (400 mM) of external ethanol. For (A) significance was assessed by Kruskal-Wallis one-way analysis of variance with post-hoc comparisons; $^*P<0.05$; $N = 15$ (Wt), 15 (R39C), 15 (E465K) and 15 (R39C/E465K). For (B) significance was assessed by one-way analysis of variance with post-hoc comparisons; $^*P<0.05$; $N = 15$ (Wt), 15 (R39C), 15 (E465K), 15 (R39C/E465K).

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Expressing either wild-type (Wt) or R39C unc-18 in lof rab-3 had no effect on this rab-3 phenotype. Surprisingly, expression of unc-18 E465K was dominant to the effects of lof rab-3. Expression of the double mutant showed that the addition of the R39C mutation did not alter the dominant effect of E465K (Figure 4B). These experiments demonstrate that at low concentrations of ethanol, the lof rab-3 phenotype is dominant to both of the R39C and E465K unc-18 mutations whereas at high concentrations, E465K is dominant to rab-3 whereas rab-3 is dominant to R39C.

Exocytotic phenotypes of point mutations in lof rab-3

The E466K mutation enhances the interaction between Munc18 and Rab3 [22], without affecting syntaxin binding [21]. Despite this biochemical characterisation, the effect of the mutation on sensitivity to high concentrations of alcohol was surprisingly independent of functional rab-3. We tested whether any of the unc-18 mutations required rab-3 to affect exocytosis. We verified that lof rab-3 (y250) worms were resistant to aldicarb in comparison to Bristol N2 wild-types (Figure 5) as has been previously reported [19]. Expression of wild-type unc-18 in the lof rab-3 background had no effect on rab-3-dependent resistance to aldicarb. Despite dominant effects to lof rab-3 in alcohol sensitivity the E465K mutation had no effect on the aldicarb phenotype. The unc-18 R39C mutation, which on its own caused a mild resistance to aldicarb, was able to block partially the effects of lof rab-3 (Figure 5). Thus, despite lof rab-3 being dominant to R39C in sensitivity to alcohol the reverse was true for sensitivity to aldicarb. The R39C/E465K double mutant was not different from lof rab-3 indicating that the effects of R39C alone were suppressed by the additional E465K mutation.

Discussion

This paper demonstrates that the genetic interactions between two exocytotic proteins, unc-18 and rab-3, are different depending on the phenotypic context. For the alcohol phenotype, either the R39C or E465K unc-18 mutations increased sensitivity. The R39C mutation is characterised to decrease binding to closed conformation syntaxin for mammalian Munc18 

in vitro [24] and in vivo [36] as well as C. elegans UNC-18

in vitro [23]. This then potentially implicates this interaction with syntaxin as an important regulator of alcohol sensitivity. Although this hypothesis has not been directly tested for ethanol specifically, syntaxin hypomorphs in C. elegans do have reduced sensitivity to volatile anaesthetics [37] emphasizing a potential convergence of cellular effectors of various anaesthetics at the presynaptic terminal. On the other hand, the E465K mutation acts to increase Rab3 binding, at least for Munc18 [22]. Applying the same logic of R39C and syntaxin, this would imply that increased ethanol sensitivity of the E465K mutation would be a consequence of increased Rab3 binding. Rab3 itself does not associate with Munc18 when it is syntaxin bound [22]. Consequently the ethanol phenotype of E465K alternatively could be a secondary consequence of the reduction in syntaxin binding in favour of Rab3. This interpretation could also explain the lack of additivity of the double mutant.

The results of these mutations in the lof rab-3 genetic background, however, argue against the simple interpretation
Figure 4. The E465K unc-18 point mutation suppresses the rab-3-dependent resistance to alcohol. Loss-of-function (lof) rab-3 (y250) worms were created to express transgenically wild-type (Wt) unc-18 or unc-18 with the indicated mutations. (A) Expression of either Wt or mutant unc-18 in a lof rab-3 genetic background did not alter acute sensitivity to external ethanol at stimulatory levels (21 mM). Significance was assessed by one-way analysis of variance; $P=0.46$; $N=29$ (N2), 37 (rab-3), 31 (Wt), 31 (R39C), 31 (E465K) and 31 (R39C/E465K). (B) In comparison to Bristol N2 controls, lof rab-3 worms had decreased acute sensitivity to external ethanol at inhibitory levels (400 mM). Expression of either unc-18 E465K or R39C/E465K, but not R39C, was dominant to rab-3. Significance was assessed by Kruskal-Wallis one-way analysis of variance on ranks with post-hoc comparisons; *$P<0.05$; $N=33$ (N2), 40 (rab-3), 30 (Wt), 31 (R39C), 31 (E465K) and 31 (R39C/E465K).

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that the effects are solely the result of the same syntaxin interaction. For the stimulatory ethanol phenotype, the effects of R39C or E465K mutations were blocked. For the depressive ethanol sensitivity phenotype the E465K mutation is dominant to lof rab-3 whereas R39C is not. This then indicates that whatever the E465K mutation is doing at high ethanol concentrations, it acts both downstream and independent of functional rab-3, which itself is downstream of R39C. Interestingly, the E465K mutation is modelled on a Sly1p (yeast Sec1/Munc18 protein) that bypasses the requirement for a functional Rab protein during ER to Golgi vesicle trafficking [38]. This mutation then also bypasses the requirement of a functional Rab protein in alcohol sensitivity as expression of E465K in the lof rab-3 genetic background eliminates the rab-3 phenotype. What then are these unc-18 mutations or lof rab-3 doing to alter ethanol sensitivity? Previous work has excluded the interpretation that ethanol sensitivity is a simple reflection of alterations in signalling strength [8-10]; yet, both unc-18 and rab-3 are characterised primarily as exocytotic proteins involved potentially in docking, priming and fusion itself [13,18]. It remains possible that the action of ethanol presynaptically is at the level of synaptic vesicle trafficking or exocytosis that is separate from signalling strength per se. Alternatively, the action of ethanol could be postsynaptic and lof rab-3 or the unc-18 mutations are altering the trafficking of postsynaptic receptors whose function is modulated by ethanol. Indeed, ethanol can affect many neurotransmitter receptors including GABA (γ-aminobutyric acid), glutamate and serotonin [7]. The precise synaptic location of action of ethanol and the roles of exocytotic proteins therefore remains to be determined in greater detail. Despite this, it is clear that the unc-18 E465K mutation acts independently and can circumvent the requirement of functional rab-3 in ethanol sensitivity.

The epistatic interactions between unc-18 and rab-3 that determine ethanol sensitivity stand in direct contrast to those for signalling strength. At the worm neuromuscular junction, the R39C mutation induced resistance to aldicarb implying a reduction in signalling strength. The R39C mutation has been previously shown to increase evoked postsynaptic currents in Drosophila [31] which may be a result of an increase in initial fusion rate [28]. The total amount of neurotransmitter released per exocytotic event, however, is concurrently decreased by the R39C mutation in bovine adrenal chromaffin cells [24] which would explain the observed reduction in signalling strength as assayed by aldicarb sensitivity in C. elegans. Contrary to ethanol sensitivity, R39C unc-18 is partially dominant to lof rab-3. Indeed as the R39C mutation is itself resistant to the effects of aldicarb in comparison to wild-type unc-18, it is possible that R39C is completely dominant to lof rab-3 for aldicarb sensitivity. It is most likely that this mutation overcomes the loss of functional rab-3 in exocytosis via changes to vesicle recruitment. Null unc-18 worms have a reduction in docked vesicles [30] that is dependent on syntaxin binding [39] and lof rab-3 alleles also reduce both the total number of synaptic vesicles and their trafficking [19]. Indeed the role of Munc18 in docking is downstream of Rab3 in adrenal chromaffin cells [40]. The data here support the notion that inhibiting the closed-conformation syntaxin interaction, and hence supporting binding of Munc18/UNC-18 to open syntaxin,
helps to bypass partially the requirement of Rab3 in determining strength of neurotransmitter release.

The Munc18 E466K mutation acts to increase Rab3 binding and the number of fusion events from bovine adrenal chromaffin cells [21]. Therefore, the lack of effect of the orthologous mutation (unc-18 E465K) in the lof rap-3 genetic background could be relatively easy to rationalise. Indeed the rap-3 (y250) allele produces no detectable Rab-3 protein [19]. The phenotypic effect of the R39C mutation, however, is blocked in the R39C/E465K double mutant expressed in the lof rap-3 genetic background suggestive of an additional functional role of the E465K mutation. At present, no other biochemical effects of the E465K mutation are known [21,22]. Nonetheless, in contrast to ethanol sensitivity, the aldicarb data indicate that that the R39C mutation acts downstream and independently of rap-3, which itself is potentially downstream of E465K.

The phenotypic effects presented here are likely to be consistent with phenotypic effects in mammals. Indeed, the pleiotropic action of alcohol in mammals is conserved for many phenotypes in nematodes [41]. Mutations that affect ethanol sensitivity in nematodes have been consistently demonstrated to alter more complex alcohol phenotypes in mice, including Munc18 and Rab3 [8-11,42,43]. In fact, various GTPases have been linked with addiction in general [44-47]. Whether Munc18/UNC-18 itself is acting as an effector of Rab3 is a potential hypothesis requiring more investigation. For the exocytosis phenotypes, key insights have been derived from C. elegans, as the vast majority of exocytic proteins have orthologues in nematodes [48]. The interactions between Munc18/UNC-18 and Rab3 have thus far been only investigated with respect to exocytosis [22,40,49] and this study furthers this knowledge by showing that the unc-18 R39C mutation can overcome the secretory defects associated with lof rap-3. In addition, genetic interactions between unc-18 and rap-3 in alcohol sensitivity determined that, for this phenotype, the unc-18 E465K mutation eliminated a requirement of rap-3. Most surprisingly, we demonstrate that the epistatic interactions between mutants of unc-18 and rap-3 are distinct depending on the phenotypic context such that the R39C mutation acts downstream of Rab3 in exocytosis whereas it acts upstream of Rab3 in ethanol sensitivity. Finally, our data emphasises that simple modulation of synaptic strength is unrelated to sensitivity to ethanol and that the functional actions of alcohol are a complex cellular mechanism involving a large spectrum of neuronal proteins.

Materials and Methods

Molecular biology

All point mutations of the unc-18 rescuing construct were introduced by site-directed mutagenesis using either the GeneTailor (Invitrogen) or QuikChange (Stratagene) methods as described previously [10,23].

Nematode culture, strains and microinjection

C. elegans strains were grown and maintained on nematode growth medium (NGM) plates at 20°C with Escherichia coli OP 50 as a food source as previously described[10,23]. Strains used in this study were: Bristol N2 (wild-type reference), unc-18 (e81) and rap-3 (y250). Transgenic worms were generated by germline injection as previously described [10,23]. Transgenic expression constructs carried unc-18 cDNA, either wild-type or the indicated point mutations, under the control of its own genomic flanking regions. Successful transgenic expression was verified by coinjection with a sur-5::GFP marker (pTG96) (kind gift of Prof. A. Fire, Stanford, CA). The concentration of injected DNA was made up to 100 ng/µl with empty pBlue Script SK+ vector for all injections. For each transgenic construct, 3-5 individual independently-derived lines were generated and analysed. Results presented here were consistent for all generated lines.

Behavioural assays and analysis

All behavioural assays were performed in a temperature controlled room at 20°C using young adult hermaphrodite animals from sparsely populated plates. Locomotion rate was quantified by measuring thrashing in 200 ul Dent’s solution (140 mM NaCl, 6 mM KCl, 1 mM CaCl2, 1 mM MgCl2, and 5 mM HEPES; pH 7.4; with bovine serum albumin at 0.1 mg/ml) over a 1 minute period as described previously [10,23]. A thrash was defined as one complete movement from maximum to minimum amplitude and back again. For ethanol experiments, measurements of locomotion were made after 10 minutes exposure and are expressed as a percentage of mean locomotion rate in 0 mM ethanol measured each day (at least 10 control animals per transgenic line). Animals were assessed in both low ethanol concentrations that stimulate locomotion (21 mM) and high ethanol concentrations that depress locomotion (400 mM) [8,10,25]. All data are expressed as mean ± S.E. Significance was tested by one-way analysis of variance (ANOVA) and post-hoc comparison of means using either the Student-Newman-Keuls test or Dunn’s test (where samples sizes were unequal). Aldicarb sensitivity was determined by measuring time to paralysis following acute exposure. For each experiment, 20-25 worms were moved to NGM plates containing aldicarb (1 mM; Sigma Chemical) and assessed for paralysis every 10 or 30 minutes after drug exposure by mechanical stimulation of the worms with a thin tungsten wire. Significance was tested by two-way ANOVA and post-hoc comparison of means using the Student-Newman-Keuls test. Experiments were performed three times.

Author Contributions

Conceived and designed the experiments: JRJ JWB. Performed the experiments: JRJ SK KR JWB. Analyzed the data: JRJ SK KR JWB. Wrote the manuscript: JWB.
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