Ethanol

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Abstract

Ethanol and general anesthetics are widely used drugs, but the mechanism of action of these compounds has remained uncertain despite intensive study. Biochemical and electrophysiological experiments have shown that these compounds alter the function of a large variety of receptors, ion channels, transporters and second messenger systems at physiological concentrations. Yet, the contribution of any of these potential targets to the intoxicating or behavioral effects of the drugs is unclear. Unbiased forward genetic screens for resistant or hypersensitive mutants represent an attractive means of identifying the relevant molecular targets or biochemical pathways mediating the behavioral effects of neuroactive compounds. *C. elegans* has proven to be a particularly useful system for such studies. The behavioral effects of ethanol and certain general anesthetics occur at equivalent tissue concentrations in mammals and in *C. elegans*, suggesting the existence of conserved drug targets in the nervous system.

This chapter reviews the results of studies directed toward determining the mechanisms of action of ethanol and general anesthetics. Studies of the neural adaptations that occur with prolonged drug exposure are also discussed. The methods used to characterize the actions of ethanol and anesthetics should be applicable to the characterizations of other compounds that affect the behavior of *C. elegans*.

Introduction

Ethanol and volatile anesthetics are widely used drugs that have profound behavioral effects. Nevertheless, the fundamental mechanisms underlying these behavioral responses have remained uncertain. Similar intoxicating effects of these compounds are observed in invertebrates and mammals suggesting the existence of conserved targets in the nervous system.

Ethanol and anesthetics affect most behaviors of *C. elegans*. The neurodepressive properties of these compounds have provided the basis for efficient screens for resistant or hypersensitive mutants. Molecular characterization of these mutants has led to the identification of likely drug targets and biochemical pathways mediating the behavioral effects of these compounds.

Behavioral Responses of *C. elegans* to Ethanol

C. elegans exhibits obvious behavioral changes upon acute exposure to ethanol. Exogenous ethanol produces reversible, dose-dependent effects on the body bends responsible for locomotion, speed of movement and frequency of egg laying (Davies et al., 2003: PMID14675531). Progressive flattening of the body-bend amplitudes becomes apparent at 100-200mM ethanol and complete flattening occurs at 400-500 mM exogenous ethanol. The changes in the amplitude of the body bends correlate with a progressive decrease in the speed of movement, as determined by image analysis (Fig 1). A similar dose-response curve is observed for egg-laying behavior when animals are maintained on agar plates. Nearly complete inhibition of both locomotion and egg laying occur at an exogenous dose of 400-500 mM ethanol. This corresponds to an internal or tissue concentration of 22-29 mM ethanol. Ethanol concentrations can readily be measured in whole animal extracts following a typical acute exposure (22 min). The concentrations are comparable to those causing intoxication in other systems. For instance, 0.1% ethanol, a common legal drinking limit, corresponds to 21.7 mM blood ethanol. Blood and brain ethanol concentrations are the same in humans. At 400-500 mM ethanol, *C. elegans* is still capable of movement on plates when stimulated, although the animals are uncoordinated.

The effect of higher concentrations of ethanol on the movement of *C. elegans* in liquid has also been studied. (Morgan and Sedensky, 1995: PMID8749805). Animals exhibited a steady state response to ethanol after 5 minutes of exposure. Mobility was scored for 10 sec at each concentration of ethanol after 5 minutes of exposure. EC50s were defined as the concentration in which 50% of the animals were immobile for greater than 10 sec. Under these conditions, the EC50 of N2 was 1050 +/- 30 mM exogenous ethanol. Again, the immobility was reversible. This higher concentration required to inhibit movement in liquid may correspond to an anesthetic dose of ethanol in *C. elegans*.

Loss of *slo-1* Function Results in Strong Resistance to Ethanol

Genetic screens for mutants resistant to the behavioral effects of ethanol on locomotion and egg laying led to the isolation of 29 mutants belonging to nine complementation groups (Davies et al., 2003: PMID14675531). All of the mutants exhibiting strong resistance to the effects of ethanol on these behaviors were found to have mutations in the

same gene, *slo-1*. *slo-1* encodes a *C. elegans* homologue of the mammalian BK potassium channel (Wang et al., 2001: PMID1738032). Mutations in *slo-1* result in uniquely strong resistance to the effects of ethanol on both locomotion and egg laying suggesting a special relationship between BK channels and ethanol sensitivity.

The resistance of *slo-1* mutants appears relatively specific. No resistance was found to other compounds that inhibit locomotion including muscimol and serotonin. No differences in the metabolism or internal concentrations of ethanol were found in *slo-1* mutants, suggesting that the resistance was not due to diminished penetration of ethanol or increased ethanol metabolism. *slo-1* mutations are thought to result in hyperactive neurotransmission, reflected as increased sensitivity to the acetylcholinesterase inhibitor, aldicarb. However, mutants with much stronger aldicarb hypersensitivity, such as *goa-1(n363)* and *dgk-1(nu62),* show no or comparatively little ethanol resistance. Together with the genetic results these findings suggested that the uniquely strong resistance of *slo-1* was not an indirect effect.

Ethanol Activates the SLO-1 Channel In Vivo

Recordings from *C. elegans* neurons in vivo revealed that ethanol causes an increase in a SLO-1-dependent current (Fig 2). This effect occurs at doses of ethanol that cause intoxication in *C. elegans* as well as in mammalian systems. No effect of ethanol on whole-cell currents was observed in *slo-1* mutants. Ethanol increased the frequency of SLO-1 channel openings in excised patches, but did not alter the size of the conductance. The ethanol effects on single channel activity occur in the absence of cytosolic factors, consistent with a direct effect of ethanol on the BK channel. Similar effects of ethanol on *C. elegans* SLO-1 channels can be observed in recordings from multiple different neurons including sensory neurons, interneurons and motorneurons. Activation of SLO-1 channels should inhibit neuronal excitability and decrease neurotransmitter release, possibly explaining the neurodepressive properties of ethanol.

Analysis of gain-of-function mutations in *slo-1* confirmed the role of SLO-1 activation in ethanol sensitivity. Recordings from two gain-of-function mutants, *ky389gf* and *ky399gf*, revealed increases in the frequency of SLO-1 channel opening, similar in magnitude to that produced by intoxicating concentrations of ethanol. The *ky389gf* and *ky399gf* mutations result in similar depressive behavioral effects as those generated by ethanol, including comparable decreases in speed of locomotion and egg laying frequency. Hence, a selective increase in SLO-1 channel activity could recapitulate behavioral effects of ethanol. Although the role of BK channel activation in ethanol intoxication in mammals is not fully understood, similar ethanol-induced increases in the frequency of opening of mammalian BK channels have been observed in vitro (Dopico et al., 1998: PMID9435186).

Ethanol Hypersensitive Mutants

Ethanol hypersensitive mutants of *C. elegans* have also been described (Morgan and Sedensky, 1995: PMID8749805). A set of mutants originally identified on the basis of hypersensitivity to volatile anesthetics (see below) was tested for altered ethanol sensitivity. At higher doses, ethanol can act as an anesthetic. Testing was completed at

the higher concentrations of ethanol required to inhibit movement in liquid. Several of the mutants that were hypersensitive to volatile anesthetics also showed hypersensitivity to ethanol in this assay. One of these mutants, *unc-79*, was resistant rather than hypersensitive to ethanol. The mechanism of the altered responses to ethanol in this class of mutants is unclear.

npr-1 Regulation of Ethanol Tolerance

As in other systems, acute behavioral adaptation (acute tolerance) occurs in *C. elegans* (Davies et al., 2004: PMID15182714). Adaptation occurs in most systems provided that tissue concentrations of ethanol remain relatively constant. It is believed that the adaptation to ethanol represents plasticity of the nervous system or a mechanism that compensates for the physiological effects of the drug. In *C. elegans*, adaptation to the effect of ethanol on locomotion occurs in the absence of any change in the internal or tissue concentration of ethanol (Fig 3). Adaptation occurs more rapidly in CB4856 (a wild strain isolated in Hawaii) than N2. These differences in the rate of development of acute tolerance were found to be due to allelic variation in a single gene, *npr-1*. *npr-1* encodes a NPY-like receptor protein (de Bono and Bargmann, 1998: PMID9741632). Allelic variation in *npr-1* had previously been shown to account for differences in food-dependent behaviors, including social (CB4856) verses solitary (N2) feeding. CB4856 has a lower function allele of *npr-1* than the allele found in the N2 strain. Multiple wild isolates are known to have either the N2 or the CB4856 allele of *npr-1*. When these isolates were tested, the relative rate of adaptation to ethanol was predicted in all cases by the particular allele of *npr-1*. More rapid ethanol tolerance was also observed in animals carrying a loss of function allele of *npr-1* in an otherwise N2 background. A known suppressor, *ocr-2(ok47)*, of the food-dependent clumping behavior of *npr-1* loss-of-function mutants did not suppress the more rapid adaptation to ethanol suggesting that the functions of *npr-1* in ethanol tolerance and food-dependent behavior are separable. Replacing *npr-1* function in different subsets of *npr-1*-expressing cells indicated that *npr-1* acts in different neurons or a larger subset of neurons for the modulation of ethanol tolerance compared with those neurons required for social behavior.

Although it is unclear whether *npr-1*-related genes play a role in natural variation in ethanol responses in higher systems, there is evidence for a role of NPY signaling pathways in acute tolerance to ethanol in rodents. Knockout mutants of the NPY-encoding gene and the NPY Y1 receptor-encoding gene each results in more rapid tolerance to ethanol (Thiele et al., 1998: PMID9845072) (Thiele et al., 2002: PMID11826154)

Ethanol Induced Changes in Gene Expression

Kwon et al. (2004) (PMID 15028283) have taken a genomics approach to understanding some of the actions of ethanol and the response of the organism. They used wild-type worms treated with an anesthetic dose of ethanol and collected RNA at various time points during the ethanol treatment. They used that RNA to probe microarrays that represent almost all of the predicted *C. elegans* open reading frames to determine where and when changes in gene expression occurs during ethanol treatment. They identified 230 genes that showed altered expression during the treatment, approximately 1% of the

genes in C. elegans (Kwon et al., 2004; PMID 15028283). Most of these changes were found to occur by the 6 hour time point although some genes were found to respond in as little time as 15 minutes. *slo-1* and *npr-1* were not identified as genes that showed changes in expression; this suggests that whatever response the worm makes to compensate for the presence of ethanol in its system, it is not through direct changes in expression levels of those two genes that have been shown by mutant analysis to affect ethanol responses.

Behavioral Responses of *C. elegans* to Volatile Anesthetics

The effects of volatile anesthetics on multiple behaviors of *C. elegans* have been determined (Morgan and Cascorbi, 1985: PMID4003794) (Crowder et al., 1996: PMID8873562). In vertebrates, anesthesia is often defined as a failure to respond to a noxious stimulus. Dose response curves have been determined in *C. elegans* for multiple effects of the anesthetics halothane, isoflurane and enflurane (Table 1). Most behaviors were scored in sealed glass chambers at 20-22 degrees Celsius. Recovery was assayed on fresh plates in room air for at least 16 hours. Immobility (defined as a lack of observable movement for 5 or 10 seconds) occurs at a concentration of 3.2 to 3.5 vol% halothane or a calculated aqueous concentration of about 2.5 mM at 20 degrees. This is higher than the concentration required for anesthesia in humans (approximately 0.21 mM). It is possible that somewhat different targets are required for complete immobility in *C. elegans*. Assays based on complete disruption of other behaviors in *C. elegans* reveal anesthetic sensitivities that are equivalent to that required for anesthesia in humans. Crowder et al. determined dose response curves for the effects of halothane on many behaviors including chemotaxis, coordinated movement, defecation, pumping, egg laying and mechanosensation. As seen in Table 1, the effects of halothane can be divided into those occurring at low, intermediate and high concentrations. Male mating behavior, chemotaxis, and coordinated movement were all completely disrupted at essentially the same low anesthetic concentration that produces anesthesia in humans. Mechanosensation, egg laying, pumping and defecation can be considered intermediately sensitive. As indicated, comparatively high concentrations were required for complete immobility or disruption of gross movement. The rapid time course for the effect of halothane on most behaviors is consistent with action of the anesthetic on neural signaling. Essentially full recovery was observed after exposure to 1 vol% halothane; whereas, recovery was incomplete after exposure to immobilizing concentrations.

Mutants Hypersensitive to Volatile Anesthetics

The molecular mechanisms responsible for the anesthesia produced by volatile anesthetics are uncertain. Genetic studies in *C. elegans* have provided clues to the possible mechanisms of action of these drugs. A number of mutants hypersensitive to the immobilizing effects of anesthetics have been identified (Morgan and Sedensky, 1994: PMID7943840). *unc-79* and *unc-80* greatly increase sensitivity to certain volatile anesthetics including halothane, chloroform, methoxyflurane, and thiomethoxyflurane. Slight resistance or no change in sensitivity was observed to other anesthetics including enflurane, isoflurane, fluroxene and flurothyl. An additional six hypersensitive mutants have been identified that exhibit hypersensitivity to either all anesthetics tested or specifically to enflurane, isofluane and diethylether.

In a search for suppressors of the *unc-79* hypersensitive phenotype, *unc-1* was identified as a strong suppressor (Humphrey e al., 2002: PMID12015284). *unc-79;unc-1* is similar to N2 in its responses to most volatile anesthetics. *unc-1* loss-of-function mutations are identical to N2 in anesthetic responses with the exception of approximately 30% hypersensitivity to diethyl ether and slight resistance to halothane. A dominant allele of *unc-1(n494)* shows increased sensitivity to halothane, isoflurane, enflurane and ether. *unc-1* encodes a protein with extensive homology to human stomatin. Mammalian stomatin binds to quinolines and other lipophilic molecules. Loss of stomatin from erythrocytes is the cause of overhydrated hereditary stomatocytosis, a condition characterized by anemia, increased red blood cell permeability to Na+ and K+, and swelling/lysis of RBCs. Stomatin is thought to regulate an ion channel. *unc-1* is broadly expressed in the *C. elegans* nervous system and interacts with a class of epithelial sodium channels (ENaCs). Mutations in one of these channels, *unc-8*, result in effects on anesthetic sensitivity similar to those observed in *unc-1* mutants. Stomatin and stomatin-like proteins are thought to be localized to lipid microdomains in cell membranes, termed lipid rafts. Such rafts may localize multiple membrane proteins into complexes. A number of proteins that have been postulated as targets of volatile anesthetics are associated with lipid rafts including ligand-gated channels, SNARE complex members and G protein-coupled receptors.

Molecular characterization of an additional halothane hypersensitive mutant (*fc21*) revealed a mutation in the gene *gas-1* which encodes a subunit of complex I of the mitochondrial electron transport chain of *C. elegans* (Kayser et al., 2004: PMID15277919). Electron transport and complex I dependent oxidative phosphorylation in isolated mitochondria are decreased by the *gas-1(fc21)* mutation. Halothane accumulates in the mitochondria of both *C. elegans* and mammals. It is unclear if volatile anesthetics cause anesthesia through inhibition of mitochondrial function. The *gas-1* results are nevertheless intriguing as patients with mitochondrial disease show altered sensitivity to volatile anesthetics and individuals with certain complex I deficiencies can be very sensitive to anesthetics such as sevoflurane.

A Neomorphic Syntaxin Mutation Causes Resistance to Volatile Anesthetics

A large screen, 500,000 genomes, for mutants resistant to the immobilizing effects of halothane was completed but did not result in the isolation of a resistant strain. Changes in multiple genes may be required for such resistance. A genetic analysis of the lower dose effects of halothane and isoflurane on coordinated movement has also been pursued (van Swinderen et al., 1999: PMID10051668). By screening existing mutants, the *md130* strain, which contains a mutation in the neural syntaxin gene (*unc-64*), was found to have marked resistance to both isoflurane and halothane. *md130* animals move with a normal waveform and at near normal velocity in the absence of anesthetic. As measured in a dispersal assay, over 2.5% isoflurane was required for a significant change in the dispersal of *md130* compared to 0.7% for wild type animals. The resistance to halothane was also significant requiring 1% for a significant change in *md130* verses 0.2% for wild type. The *md130* mutant is therefore highly resistant to concentrations of anesthetics that are clinically relevant. In fact no other metazoans have been described with a

comparable level of resistance. The phenotype of *md130* appears to be rare in *C. elegans*. A screen of 6,600 haploid genomes failed to reveal similarly resistant mutants, as did testing of an additional 70 existing strains.

The mechanism of anesthetic resistance of *md130* is complex and uncertain. The volatile anesthetic phenotype of *md130* is semidominant. *md130* behaves as a gain-of-function mutant for the anesthetic resistance but as a reduction-of-function mutant for its locomotion phenotype (mildly Unc) in the absence of anesthetic. *md130* was originally isolated based on its resistance to aldicarb, an acetylcholinesterase inhibitor. For aldicarb resistance, *md130* also behaves as a reduction-of-function mutation. Other reduction-of-function mutations in syntaxin cause hypersensitivity to halothane and isoflurane, rather than resistance. Similarly, mutations in other genes functioning in exoctyosis (SNAP-25 and synaptobrevin) lead to hypersensitivity to volatile anesthetics. These effects are consistent with a presynaptic inhibitory effect of volatile anesthetics. Indeed, anesthetics themselves cause resistance to aldicarb but not levamisole in *C. elegans*.

Sequencing of the *unc-64* gene in *md130* revealed a missense mutation at a splice donor site of the sixth intron. Both wild type and truncated protein products were found by reverse transcription and PCR of *md130* RNA. The anesthetic resistance of *md130* can be explained by a model where the truncated syntaxin product antagonizes the action of anesthetics against another protein(s) to which syntaxin binds.

Natural Variation in Anesthetic Sensitivity

Natural variation in volatile anesthetic sensitivity has been examined in *C. elegans* recombinant inbred (RI) strains (van Swinderen et al., 1997: PMID9223344). The analyzed behaviors included coordinated movement, mating ability and paralysis. Significant changes in halothane sensitivity were observed in these strains. This included resistance (up to 2 fold) for effects of halothane on coordinated movement. The genetic determinants for mating anesthesia and dispersal appeared distinct from the determinants for paralysis in the RI strains. Mapping the dispersal anesthesia and mating anesthesia traits revealed 2 QTLs reaching significance for dispersal, one on chromosome I, and one on chromosome V. A QTL on the same portion of chromosome V reached significance for mating anesthesia. A further study showed that the strongest QTL for isoflurane anesthesia mapped to the same genomic location as for halothane anesthesia on chromosome V. Mapping and positional cloning may lead to the identification of the genes defining these volatile anesthetic QTLs.

Conclusions and Prospects

Genetic studies in *C. elegans* have provided a means of understanding the mechanisms of action of ethanol and general anesthetics. Despite their profound effects on behavior, the molecular targets of these compounds have remained elusive for decades. Biochemical approaches in vitro have suggested that ethanol and volatile anesthetics can affect the activity of a wide variety of neural proteins. It has been unclear whether such effects relate to the behavioral responses to these drugs.

Many of the mechanisms identified in *C. elegans* are likely to contribute to behavioral effects of ethanol and anesthetics in mammalian systems. For instance, it is intriguing that the mammalian BK channel can be modulated by ethanol in vitro in the same way that the *C. elegans* channel is modulated in vivo. Further genetic analysis in *C. elegans* may lead to a more complete understanding of both the acute and chronic effects of ethanol and anesthetics. It may be possible to gain a molecular understanding of not only the acute behavioral responses, but also the more complex adaptations and compensatory mechanisms that are likely to be responsible for drug tolerance, sensitization, withdrawal and dependency.

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Figure 1. Behavioral responses of *C. elegans* to ethanol.
(A) Dose-response curve describing the inhibitory effects of ethanol on speed (●) and frequency of egg laying (□) of the wild-type (N2) strain. The measured internal concentration of ethanol in the animals for the 400 mM and 500 mM treatments was 22 ± 0.8 mM and 29 ± 0.5 mM respectively. Error bars, s.e.m.

(B) Ethanol-induced decrease in body-bend amplitude during locomotion. Ethanol (200 mM) causes a small decrease in the amplitude of body bends. Complete flattening of the body bends can occur at 400- and 500-mM ethanol. The effect is most pronounced on the posterior of the body (left). Scale bar, 200 µm.

Figure 2. Activation of the SLO-1 current by ethanol.

(A) Bath application of ethanol selectively and reversibly increases the magnitude of the outward-rectifying current in wild-type but not *slo-1(-)* mutant CEP neurons. Current traces for voltage steps to +86 mV holding potential on left and mean current *versus* voltage plots on right for before (○), during (●) and after (□) treatment with ethanol (EtOH).

(B) Average percent change in whole-cell current at +86 mV is plotted for different conditions and animals. Dextrose = Dex. Sample size indicated above each bar. Asterisks indicate significant changes ($P < 0.05$).

(C) Ethanol reversibly potentiates the activity of a single SLO-1 channel in an excised patch from a sensory CEP neuron held at +6 mV. Dotted lines indicate baseline levels.

(D) Recording of SLO-1 channels excised from VA motorneurons also shows reversible potentiation by ethanol (100 mM) at +40 mV holding potential.

Figure 3. Acute ethanol tolerance in *C. elegans* varies between two wild strains.

(A) The relative speed (% of the speed of untreated animals) of three strains increases during a continuous exposure to exogenous ethanol (500 mM). The wild strain, CB4856 and a CB4856-derived strain that had been outcrossed seven times to the N2 strain, and selected for a rapid development of acute tolerance at each outcross, demonstrate more rapid recovery of speed (acute tolerance), particularly in the 10–30 minute interval than does the N2 wild strain. Error bars, s.e.m.

(B) *C. elegans* has low permeability to exogenous ethanol. The internal ethanol concentration (mM) within the two wild strains, N2 and CB4856, at the 10-, 30- and 50-minute time points remains constant but CB4856 demonstrated higher internal ethanol concentrations than N2.

Table 1. Summary of Behavioral Effects of Volatile Anesthetics in *Caenorhabditis elegans*

Behavior	EC_{50} (mean ± SEM) (vol%)				Effect Time (min)	% Recovery	
	Halothane	Isoflurane	Enflurane	Desflurane		p 1%	p 4%
Mating	0.30 ± 0.02	0.63 ± 0.02	0.71 ± 0.08	4.1 ± 0.3	10	97	47
Chemotaxis	0.34 ± 0.01	0.89 ± 0.05	0.97 ± 0.08	5.1 ± 0.6	<30	100	53
Coordinated Movement	0.32 ± 0.05	0.86 ± 0.09	1.1 ± 0.07	—	<20	—	—
Defecation	1.2 ± 0.05	2.5 ± 0.14	1.9 ± 0.02	—	10	—	—
Pumping	1.8 ± 0.02	3.1 ± 0.07	3.6 ± 0.001	—	10	—	—
Egg laying	2.1 ± 0.10	—	—	—	—	—	—
Mechanosensation	2.2 ± 0.07	—	—	—	—	—	—
Gross movement	3.6 ± 0.06	6.5 ± 0.05	4.7 ± 0.04	—	120	100	88

Halothane potency/recovery for mating is efficiency of N2 males x *dpy-11(e224)*; time course is N2 males x *unc-51(e369)*; chemotaxis is against isoamyl alcohol; coordinated movement data are from radial diffusion assays; defecation data are for expulsion; p = % of recovery to air control after exposure to 1 or 4 vol% halothane.

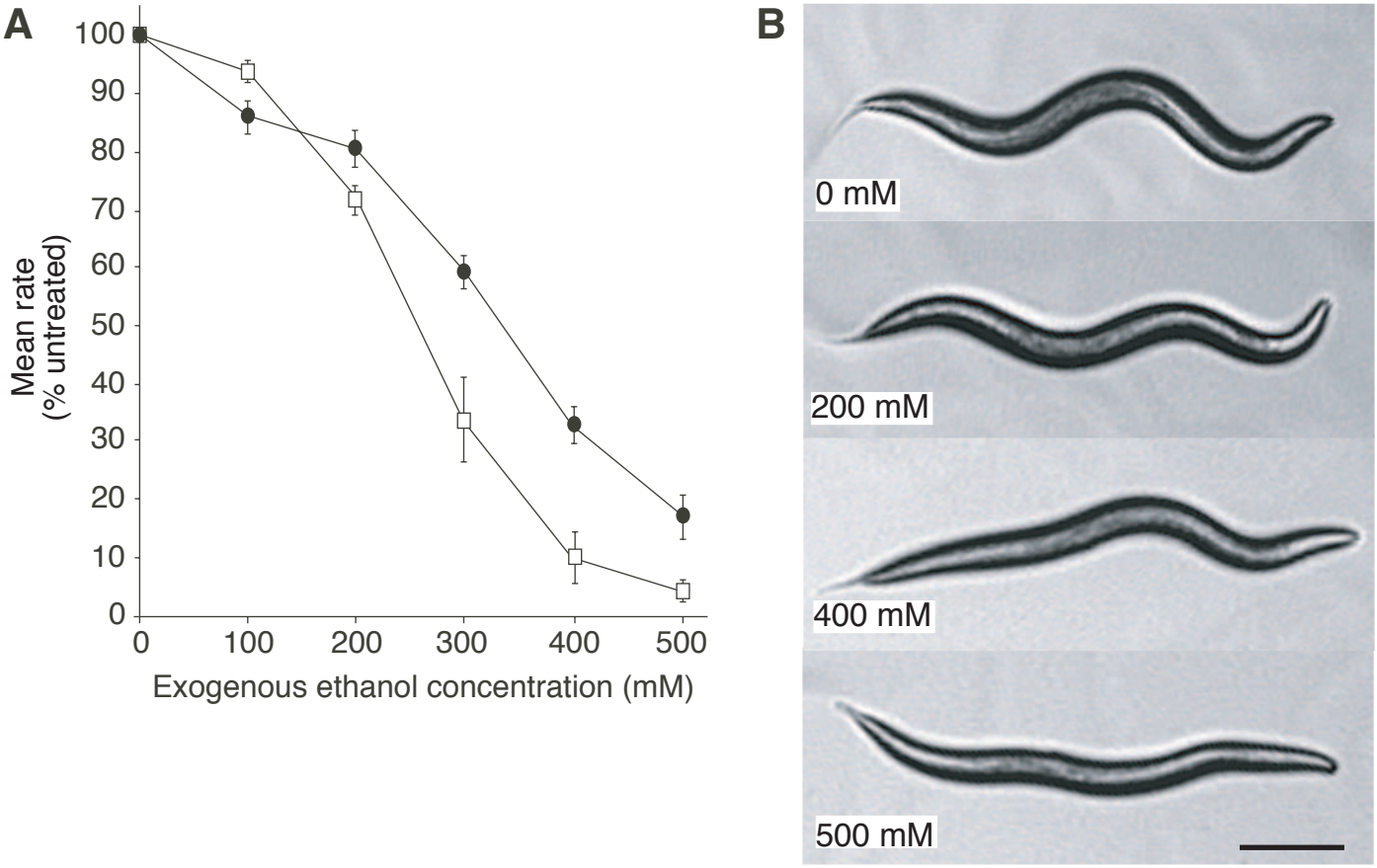
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Figure 1

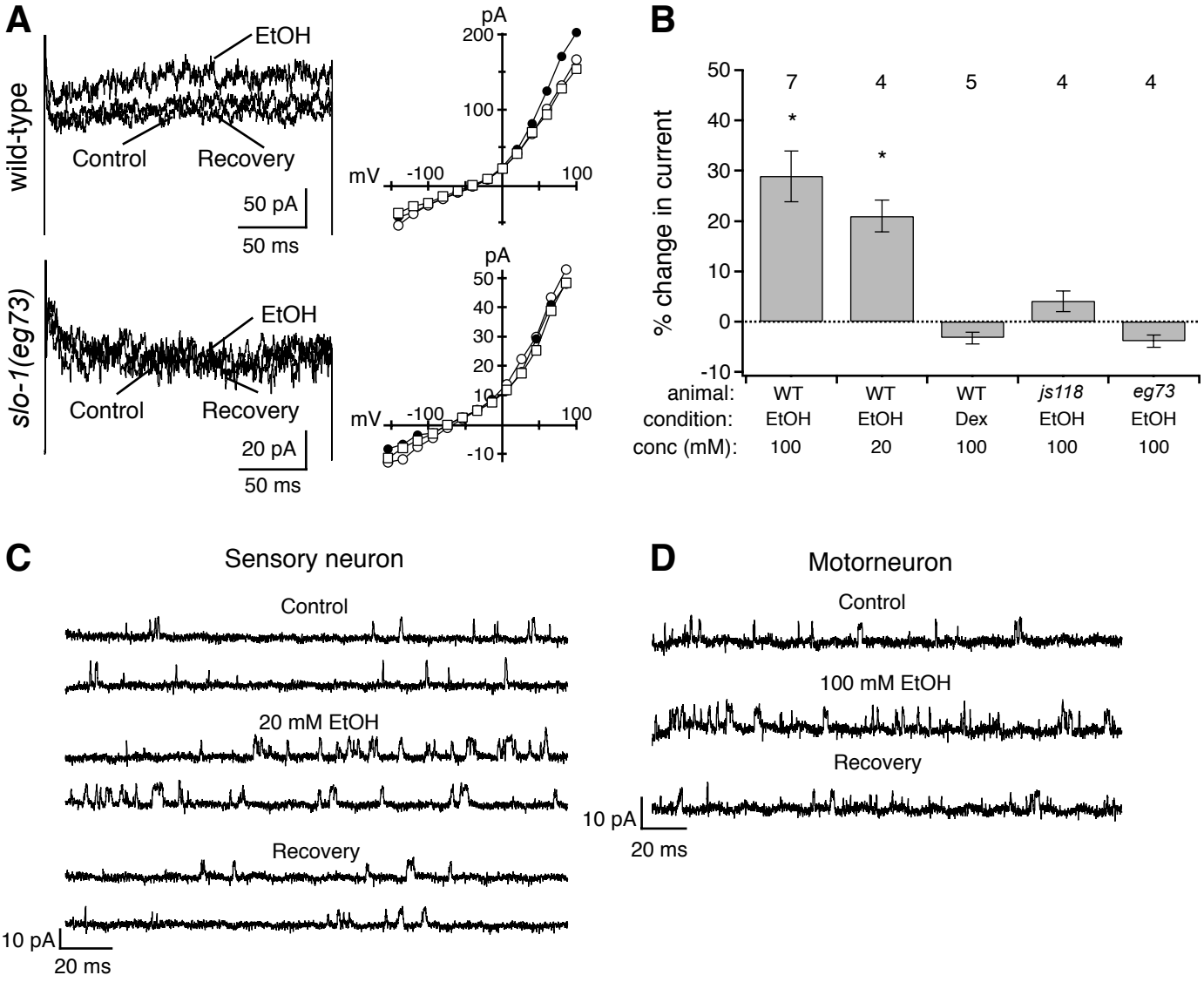
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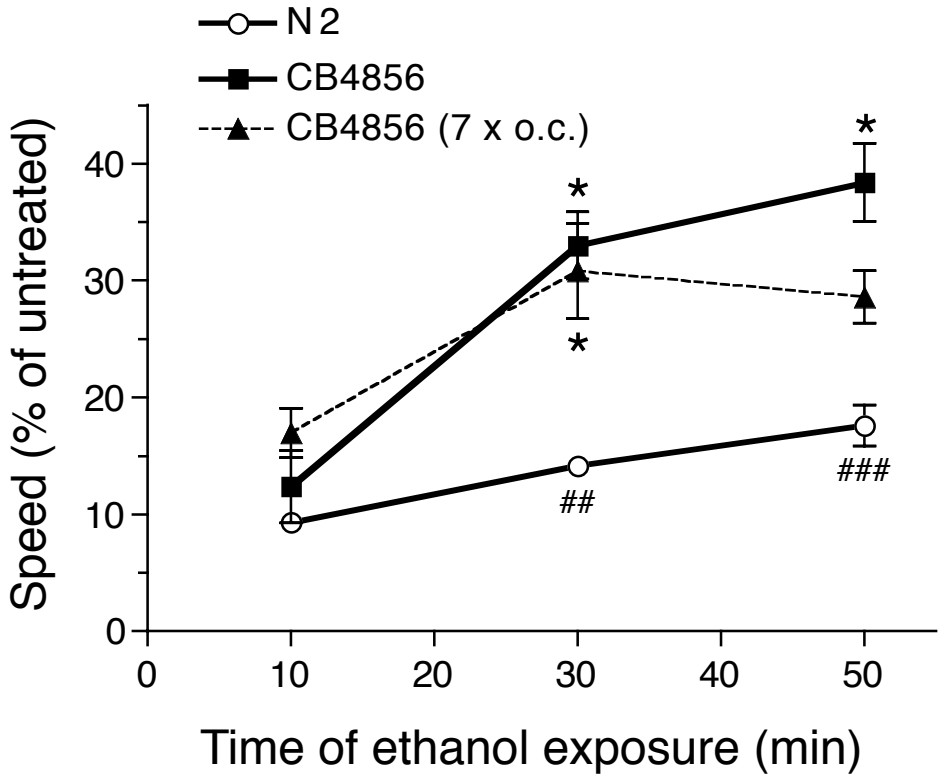
Figure 2

A



B

		Time (min)		
		10	30	50
Internal ethanol concentration (mM)	N2	26.0 ± 4.2	33.1 ± 1.4	29.5 ± 2.2
	CB4856	40.4 ± 4.1	38.2 ± 2.1	41.2 ± 1.7

Figure 3