

Video Article

Paradigms for Pharmacological Characterization of *C. elegans* Synaptic Transmission Mutants

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Abstract

The nematode, *Caenorhabditis elegans*, has become an expedient model for studying neurotransmission. *C. elegans* is unique among animal models, as the anatomy and connectivity of its nervous system has been determined from electron micrographs and refined by pharmacological assays. In this video, we describe how two complementary neural stimulants, an acetylcholinesterase inhibitor, called aldicarb, and a gamma-aminobutyric acid (GABA) receptor antagonist, called pentylentetrazole (PTZ), may be employed to specifically characterize signaling at *C. elegans* neuromuscular junctions (NMJs) and facilitate our understanding of antagonistic neural circuits.

Of 302 *C. elegans* neurons, nineteen GABAergic D-type motor neurons innervate body wall muscles (BWMs), while four GABAergic neurons, called RMEs, innervate head muscles. Conversely, thirty-nine motor neurons express the excitatory neurotransmitter, acetylcholine (ACh), and antagonize GABA transmission at BWMs to coordinate locomotion. The antagonistic nature of GABAergic and cholinergic motor neurons at body wall NMJs was initially determined by laser ablation and later buttressed by aldicarb exposure. Acute aldicarb exposure results in a time-course or dose-responsive paralysis in wild-type worms. Yet, loss of excitatory ACh transmission confers resistance to aldicarb, as less ACh accumulates at worm NMJs, leading to less stimulation of BWMs. Resistance to aldicarb may be observed with ACh-specific or general synaptic function mutants. Consistent with antagonistic GABA and ACh transmission, loss of GABA transmission, or a failure to negatively regulate ACh release, confers hypersensitivity to aldicarb. Although aldicarb exposure has led to the isolation of numerous worm homologs of neurotransmission genes, aldicarb exposure alone cannot efficiently determine prevailing roles for genes and pathways in specific *C. elegans* motor neurons. For this purpose, we have introduced a complementary experimental approach, which uses PTZ.

Neurotransmission mutants display clear phenotypes, distinct from aldicarb-induced paralysis, in response to PTZ. Wild-type worms, as well as mutants with specific inability to release or receive ACh, do not show apparent sensitivity to PTZ. However, GABA mutants, as well as general synaptic function mutants, display anterior convulsions in a time-course or dose-responsive manner. Mutants that cannot negatively regulate general neurotransmitter release and, thus, secrete excessive amounts of ACh onto BWMs, become paralyzed on PTZ. The PTZ-induced phenotypes of discrete mutant classes indicate that a complementary approach with aldicarb and PTZ exposure paradigms in *C. elegans* may accelerate our understanding of neurotransmission. Moreover, videos demonstrating how we perform pharmacological assays should establish consistent methods for *C. elegans* research.

Video Link

The video component of this article can be found at <https://www.jove.com/video/837/>

Protocol

Aldicarb Exposure Paradigm

1. On the first day, ensure that at least thirty young adult stage worms of each genotype and of each replicate will be available for aldicarb assays on the second day. It is best if an experimenter selects fifty or more L4 stage worms onto fresh NGM plates (best without nystatin), which contain *E. coli* (preferably OP50) as a food source, and grow them for 12-24 hours at a consistent and permissive temperature (20°C to 22°C is best, although 25°C is okay).
2. On the second day, make a 100 mM stock solution of aldicarb with 70% ethanol (EtOH) and 30% ddH₂O. Spread the appropriate amount of aldicarb onto NGM minus nystatin plates with defined volumes to achieve the desired aldicarb concentrations. We consistently use 0.5 mM aldicarb by plating 37.5 µL of 100 mM aldicarb onto 7.5 mL NGM plates. Allow the aldicarb plates to dry for roughly 30-60 minutes at room temperature. It is not necessary to crack the lids. Alternatively, aldicarb can be added to NGM and stored at 4°C for one week.
3. After drying, plate consistent volumes of *E. coli* (preferably OP50) onto the center of each aldicarb plate and dry for another 30-60 minutes at room temperature. We consistently plate 25 µL of OP50, which creates a sufficiently sized food lawn to keep the worms concentrated in a small spot without overcrowding.

- When the food lawn is dry, one may proceed with aldicarb assays. Due to the subjective nature of aldicarb assays, it is highly recommended that experiments be performed "blindly". A colleague of the primary experimenter could re-label the original plates with worms to be assayed. Likewise, the colleague could transfer worms from the original plates to ciphered aldicarb plates immediately before starting a timer. If the experimenter anticipates assaying a particular strain of worms with a characteristic phenotype, such as uncoordination, then there must also be a control with a similar phenotype to reduce bias. Furthermore, it is best if the experimenter assays a wild-type strain, as well as a resistant strain and a hypersensitive strain, in parallel to help standardize experiments. The experimenter should strive to analyze a consistent number of worms for each replicate. We consistently analyze thirty worms of a single genotype for each replicate. We also perform at least three replicates for each experiment. An experienced experimenter should be able to analyze at least six strains at a time.
- Count the number of paralyzed worms by prodding in a consistent manner each worm with a platinum wire. We consistently prod our worms twice on the head and twice on the tail every 30 minutes for a total of three hours. Cessation of pharyngeal pumping may also be used to define paralysis, but only if the experimenter employs a consistent definition of paralysis over all assays. Also, it is worth noting that some worms, especially those that are resistant to aldicarb, may attempt to crawl off the plate. In this case, the experimenter may spread a consistent amount of palmitic acid, a physical barrier to worm locomotion, around the aldicarb plates. We spread 25 μ L of 10 mg palmitic acid/mL EtOH.

PTZ Exposure Paradigm

- On the first day, ensure that at least thirty young adult stage worms of each genotype and of each replicate will be available for PTZ assays on the second day. It is best if an experimenter selects fifty or more L4 stage worms onto fresh NGM plates (best without nystatin), which contain *E. coli* (preferably OP50) as a food source, and grow them for 12-24 hours at a consistent and permissive temperature (20°C to 22°C is best, although 25°C is okay).
- On the second day, make a 0.5 g PTZ/mL ddH₂O stock solution of PTZ. Spread the appropriate amount of PTZ onto NGM minus nystatin plates with defined volumes to achieve the desired PTZ concentrations. Because we prefer dose-response assays for PTZ, we plate varying amounts of PTZ onto 7.5 mL NGM plates. For example, one may plate 37.5 μ L of the PTZ stock solution onto a 7.5 mL NGM plate to make a 5 mg PTZ/mL solution. Allow the PTZ plates to dry for roughly 60-120 minutes at room temperature. It is not necessary to crack the lids. Alternatively, PTZ can be added directly to NGM, but it should be immediately used for assays after solidifying. The stability of PTZ is considerably less than that of aldicarb. Therefore, assays with older PTZ plates are less reliable. PTZ should be stored at -20°C in a desiccator with a drying agent before use. We prefer to discard PTZ approximately two months after opening to ensure stability.
- After drying, plate consistent volumes of *E. coli* (preferably OP50) onto the center of each PTZ plate and dry for another 30-60 minutes at room temperature. We consistently plate 25 μ L of OP50, which creates a sufficiently sized food lawn to keep the worms concentrated in a small spot without overcrowding.
- When the food lawn is dry, one may proceed with PTZ assays. Although PTZ assays are less subjective than aldicarb assays, it is still highly recommended that experiments be performed "blindly". A colleague of the primary experimenter could re-label the original plates with worms to be assayed. Likewise, the colleague could transfer worms from the original plates to ciphered PTZ plates immediately before starting a timer. If the experimenter anticipates assaying a particular strain of worms with a characteristic phenotype, such as uncoordination, then there must also be a control with a similar phenotype to reduce bias. Furthermore, it is best if the experimenter assays a wild-type strain, as well as strains with anterior or full-body convulsions, in parallel to help standardize experiments. The experimenter should strive to analyze a consistent number of worms for each replicate. We consistently analyze thirty worms of a single genotype for each replicate. We also perform at least three replicates for each experiment. An experienced experimenter should be able to analyze at least three strains at a time. Yet, the frequency and intensity of PTZ-induced convulsions usually peak between 15-30 minutes of exposure, and then attenuate as worms become paralyzed or, in some cases, acclimate to the drug.
- Count the number of "epileptic-like" convulsing worms every 30 minutes for a total of one hour. It is best to score the number of worms with anterior convulsions, which we call "head-bobs", separately from the number of worms with full-body convulsions, full-body paralysis, which we call "tonic", or a combination of anterior convulsions with BWM paralysis, which we call "tonic-clonic". Most worms of a single genotype exhibit only one kind of convulsion. However, tonic-clonic worms often become completely tonic, so one should continue the assay past 30 minutes, even if all worms have already exhibited convulsions. Also, it is worth noting that some worms may attempt to crawl off the plate. In this case, the experimenter may spread a consistent amount of palmitic acid around the PTZ plates. We spread 25 μ L of 10 mg palmitic acid/mL EtOH.

Behavioral Responses of Selected *C. elegans* Synaptic Transmission Mutants to Aldicarb and PTZ

Mutant Name	Synaptic Role	Behavior without Drug	Behavioral Response to Aldicarb (compared to wild-type N2)	Behavioral Response to PTZ
tom-1(ok188)	inhibits synaptic transmission	uncoordinated	enhanced rate of paralysis	indistinguishable from wild-type
unc-43(n498n1186)	complex	uncoordinated	enhanced rate of paralysis	full-body convulsions
unc-25(e156)	promotes GABA transmission	uncoordinated	enhanced rate of paralysis	anterior convulsions, full-body paralysis

snb-1(md247)	promotes synaptic transmission	uncoordinated	reduced rate of paralysis	anterior convulsions
unc-4(e120)	promotes ACh transmission	uncoordinated	reduced rate of paralysis	indistinguishable from wild-type

Two neural stimulants, an acetylcholinesterase inhibitor, called aldicarb, and a GABA receptor antagonist, called pentylenetetrazole (PTZ), can be used in a complementary manner to characterize *C. elegans* synaptic transmission mutants. Excess excitatory acetylcholine (ACh) accumulates at worm body wall neuromuscular junctions (NMJs) from deleterious mutations in negative regulators of ACh transmission (e.g. tom-1 and unc-43) or positive regulators of inhibitory GABA transmission (e.g., unc-25). Conversely, excitatory ACh levels at worm NMJs are diminished by deleterious mutations in positive regulators of general synaptic transmission (e.g., snb-1) or ACh-specific transmission genes (e.g. unc-4). When compared to wild-type N2 worms, mutant worms with elevated excitatory ACh transmission at NMJs exhibit enhanced rates of aldicarb-induced paralysis, whereas mutant worms with lowered excitatory ACh transmission demonstrate reduced rates of aldicarb-induced paralysis. Although PTZ disrupts neuronal synchrony at *C. elegans* body wall muscles, not unlike aldicarb, PTZ also antagonizes inhibitory GABA at *C. elegans* head muscles. As a result, aldicarb sensitivity cannot accurately predict PTZ sensitivity. Mutant worms with specific defects in negative or positive regulation of ACh transmission are indistinguishable from wild-type N2 worms in the presence of PTZ, whereas mutant worms with defects in positive regulation of general synaptic transmission or specific defects in inhibitory GABA transmission exhibit robust PTZ-induced anterior convulsions. Moreover, unc-43 loss-of-function mutants display full-body convulsions in the presence of PTZ and likely have additional synaptic transmission abnormalities, which contribute to their unique drug responses.

Discussion

Current protocols for aldicarb exposure with *C. elegans* do not allow experimenters to distinguish between mutants with specific deficits in ACh transmission and mutants with generalized deficits in synaptic transmission, as both classes of mutants exhibit resistance to aldicarb. Likewise, aldicarb cannot be used to determine if mutants have specific deficits in GABA transmission or generalized failures to negatively regulate ACh transmission, as both classes of mutants exhibit hypersensitivity to aldicarb. Results from our PTZ exposure assays, when combined with results from aldicarb exposure assays, allows researchers to better characterize synaptic transmission mutants.

C. elegans synaptic transmission mutants may be classified in a straightforward manner by complementary aldicarb and PTZ exposure paradigms. Aldicarb resistant mutants with PTZ-induced anterior convulsions are likely deficient in general synaptic function. Conversely, aldicarb resistant mutants without PTZ-induced anterior convulsions are likely specifically deficient in ACh transmission. Mutants with aldicarb hypersensitivity, which do not exhibit PTZ-induced anterior convulsions, likely fail to negatively regulate ACh transmission. Finally, mutants with aldicarb hypersensitivity, which do exhibit PTZ-induced anterior convulsions, are likely GABA deficient.

The utility of aldicarb exposure is also weakened by its subjectivity, as different experimenters often have varying definitions of paralysis. A single experimenter's technique can also fluctuate. Also, aldicarb-exposed worms move differently in response to diverse forces of prodding. The distinction between a paralyzed worm and a responsive worm can be as subtle as a slight head or tail twitch. In addition to complementing aldicarb assays for better characterization of *C. elegans* synaptic transmission mutants, PTZ may also be used to isolate synaptic transmission mutants, especially those mutants with hypersensitivity to aldicarb. Experimenters, which utilize PTZ exposure, may simply look for anterior convulsions, instead of subtle differences in aldicarb-induced paralysis.>

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