

● PERSPECTIVE

Understanding axon guidance: attraction, repulsion, and statistical physics

Understanding axon guidance is important for developing therapies to restore neuronal connections damaged by injury or disease. Axons migrate in response to extracellular guidance molecules that induce or inhibit axon outgrowth activity within the axon. The direction of guidance is determined by the attractive and repulsive responses that the axon has to the guidance cues. In a deterministic model of guidance, the direction of guidance can be precisely determined if the attractive and repulsive effect that each cue has on the axon is known. But what if there are numerous attractive and repulsive responses induced by multiple guidance cues, and the direction of the attractive and repulsive events fluctuates? If the effect that each attractive and repulsive event has on guidance becomes too complex to measure then understanding how each molecular cue influences the guidance decision becomes impossible.

In a series of papers, we have argued that it is useful to study axon guidance as a stochastic process. This approach treats all the outgrowth activities in aggregate. Attraction and repulsion are considered as unpredictable events that occur at the molecular level. The contribution to guidance of each attractive or repulsive event becomes insignificant. Using this probabilistic approach, guidance is considered as macroscopic movement that is the product of the collective impact of all the underlying axon outgrowth events. Directionality is the product of a succession of randomly directed movement. This movement can be studied using the methods of statistical physics. In this perspective article, I explain the rationale behind this theory and its significance for understanding axon guidance.

Axon guidance, Einstein, and the random walk: The idea that extracellular molecules might guide axons was first proposed by Cajal in 1892 (Ramón y Cajal, 1892). At the time, leukocytes were known to move towards bacteria in response to diffusion gradients of bacterial toxins and Cajal imagined that neuronal growth cones could be oriented towards their target by a process similar to the “chemotactic ameboidism” of leukocytes. But it was not until the 1990s that a molecule was discovered that had the properties of a chemoattractant for axon guidance. Studies using the nematode *Caenorhabditis elegans* indicated that UNC-6, a member of the netrin protein family, was an extracellular molecule that could guide axons *in vivo* (Hedgecock et al., 1990; Ishii et al., 1992). It was also shown that biochemically isolated vertebrate netrin had chemotropic properties; when diffusible forms of the protein were presented to neurons in culture the protein could attract axons at a distance (Kennedy et al., 1994).

A few years ago, my laboratory made an interesting observation (Xu et al., 2009; Kulkarni et al., 2013). We noticed that a neuron in *C. elegans* could be tricked into generating an UNC-6 (netrin) response. We found that in animals where

UNC-6 was not present, and either the UNC-6 receptor UNC-40 (DCC) or the cytoplasmic protein UNC-53 (NAV2) were mutated, the neuron would send out an axon as if it were responding to UNC-6. Further, when we looked at many of these neurons we noticed that the direction of axon outgrowth varied. This was a surprising observation since from a deterministic point of view, an outgrowth response should only occur at the site where the external UNC-6 cue interacts with the neuron. It was not known that neurons have the intrinsic ability to polarize the axon outgrowth activity independently of the external asymmetric cue. The results also showed that neurons had the intrinsic ability to stochastically determine the direction of the axon outgrowth activity.

We noted that our observations had similarities to what is known about polarization in chemotactic cells such as yeast, *Dictyostelium discoideum*, and neutrophils (Wedlich-Soldner and Li, 2003). If chemotactic cells are exposed to a uniform concentration of a chemoattractant, the cells are still able to polarize and initiate motility, albeit in random directions. The chemoattractant apparently activates the intrinsic ability of these cells to polarize. We therefore proposed multiple UNC-40 signals; one triggers the intrinsic ability of a neuron to polarize and cause randomly oriented asymmetry, while another biases the orientation of the asymmetry relative to the external UNC-6 source (Xu et al., 2009).

One of the hallmarks of the neuron’s response to UNC-6 is that the UNC-40 receptor and other signaling molecules become asymmetrically localized to the surface membrane where the axon forms (Adler et al., 2006; Quinn et al., 2006). We observed that in the *unc-40* and *unc-53* mutants when UNC-6 is not present UNC-40 localization and axon outgrowth will occur with some probability at any one side of the neuron, however when UNC-6 is present UNC-40 localization and axon outgrowth will occur towards the UNC-6 source (Xu et al., 2009; Kulkarni et al., 2013). In these studies direction is treated as a random variable and the effects that mutations have on the probability distribution of this random variable is observed. This linked the genetic analyses to a stochastic model of guidance. The results provided a major insight; UNC-6 regulates the *probability* of axon outgrowth in each direction.

This insight, however, raised the question of how randomly directed axon outgrowth activity could cause guidance. In 1905 Albert Einstein published a landmark paper, “On the Movement of Small Particles Suspended in Stationary Liquids at Rest Required by the Molecular-Kinetic Theory of Heat” (Einstein, 1905). In this paper, Einstein developed the theoretical groundwork for measurements that could confirm the existence of atoms. Einstein gave an explanation for Brownian motion, the irregular motion of particles immersed in a fluid. It is based on a hypothesis that the particles move about because randomly moving liquid molecules continuously bombard them. Einstein realized that these impacts, and the movement of a particle over time, could be probabilistically described.

The approach Einstein used is based on a random walk model. The name “random walk” comes from a challenge to mathematically describe the walk of a man where the direction of each step is randomly chosen. Given that the direction of each step is so irregular that the next step can

not be predicted, what is the probability of the man covering a specific distance in a given time? Einstein essentially used the solution to this problem to obtain the probability of a Brownian particle covering a particular distance in time. Einstein then related the random walk of a single particle to the diffusion of many particles.

Importantly, Einstein had developed a practical explanation of physical phenomena by considering random processes. Einstein's paper had laid a foundation for stochastic modeling. Besides a Brownian particle, Einstein's approach works for the movement of many different types of objects. It allows the properties of a macroscopic system to be described even if the behavior of the system is based on the effects of numerous unpredictable, and perhaps unobservable, events.

So imagine if a migrating axon is like an inside out Brownian particle. Whereas the Brownian particle moves because stochastic forces (the impact of randomly directed liquid molecules) are driving it from the outside, the axon moves because stochastic forces (the randomly directed outgrowth activity) are driving it from the inside. At any particular time, extracellular guidance cues might interact with the axon and alter the probability of axon outgrowth activity happening at a particular side of the axon. These interactions could create directed movement.

Axon guidance as a random walk: Random walks are a tool of statistical physics. A random walk is a mathematical formalization and it uses a formal definition of randomness.

Definitions:

- (1) Random Variable—a variable that can take on a set of different possible values
- (2) Probability Distribution—possible values of a random variable and their associated probabilities
- (3) Stochastic Process—a collection of random variables
- (4) Random Walk—a mathematical formalization of a path that consists of a succession of random steps

Figure 1A shows a neuron where the direction of axon outgrowth can vary among individuals. In response to the same extracellular guidance cues in the surrounding environment, the axon in different animals will choose to grow out in a different direction; ventral (down), anterior (left), posterior (right) or dorsal (up). The direction of outgrowth can be considered a variable, X . For each direction, there is a probability that the axon will grow out in that direction. This defines the probability distribution:

<u>direction, X</u>	<u>probability</u>
ventral	$P(X = \text{ventral})$
anterior	$P(X = \text{anterior})$
posterior	$P(X = \text{posterior})$
dorsal	$P(X = \text{dorsal})$

Since the axon grows out over a period of time and time can be divided into shorter time intervals, it can be deduced that the axon outgrowth takes place through a series of steps where the direction for each step is chosen at random according to the probability distribution (**Figure 1B**).

This creates a stochastic sequence (S_n). This is a simple random walk:

$$S_n = \sum_{k=1}^n X_k$$

Evidence for the model and its implications: Considering axon guidance as a random walk shifts the emphasis from the effect that a guidance cue has on the molecular mechanisms of axon outgrowth to the effects guidance cues have on the macroscopic movement of guidance. The model makes three important predictions.

First, the direction of axon guidance is determined by the succession of randomly directed axon outgrowth activity. Axon outgrowth activity can be imagined as a series of steps over time (**Figure 1C**). This activity occurs at the molecular level. Each individual event is insignificant and may even be unobservable at the macroscopic level. However in aggregate, over time, the events can produce observable axon extension. We propose this effect can explain phenotypes we observe. For example, *unc-5* encodes an UNC-6 receptor that is thought to mediate a repulsive response to UNC-6. The HSN axon in *C. elegans* migrates towards the source of UNC-6 and in *unc-5* loss-of-function mutants the axon reaches its target. The conclusion has been that UNC-5 is not important for attraction. However, we found that there is a marked difference in the variability of the direction of axon outgrowth from the cell body in *unc-5* mutants (Kulkarni et al., 2013). Often we observe axon outgrowth that was not towards the UNC-6 source. To study how UNC-5 affects the stochastic process, we use the probability of axon outgrowth from each side of neuron in the loss-of-function *unc-5* mutant to define a probability distribution. We then use this probability distribution to simulate a simple random walk. The results show how all the guidance molecules acted together to define a directional bias for outgrowth as the axon extended from the cell body. Of course the random walk of an axon *in vivo* is not a simple walk. For one thing, the probability distribution would not remain identical as the axons moves through its environment and encounters new cues and different concentrations of the cues. The simulations provide a means to compare the directional bias created by the guidance cues in different strains. We can ask the question, how did getting rid of UNC-5 alter the directional bias from that created in a normal, wild-type animal? As **Figure 2A** shows, when UNC-5 function is missing the relative directional bias shifts. Although the HSN axon reaches its target in *unc-5* mutants, it does so with a defective guidance system.

Second, guidance cues determine the direction of guidance by combinatorial regulation. Because of the random walk, the directional response to any guidance cue is variable and depends on how all the cues are regulating the probabilities of outgrowth. This prediction is derived from probability theory. In general, the probability distribution of variable X , the direction of outgrowth, is

$$f(x) = P(X = x)$$

and it satisfies the following condition:

$$\sum_{x \in S} f(x) = 1$$

Simply stated, this means that if all the probabilities for all the possible directions are added together the sum must equal 1. This bit of probability theory has profound implications. It means that a guidance cue must affect the probability of outgrowth in more than one direction. Since the sum of all the probabilities must equal 1, if a cue increases or decreases the probability of outgrowth in one direction it must

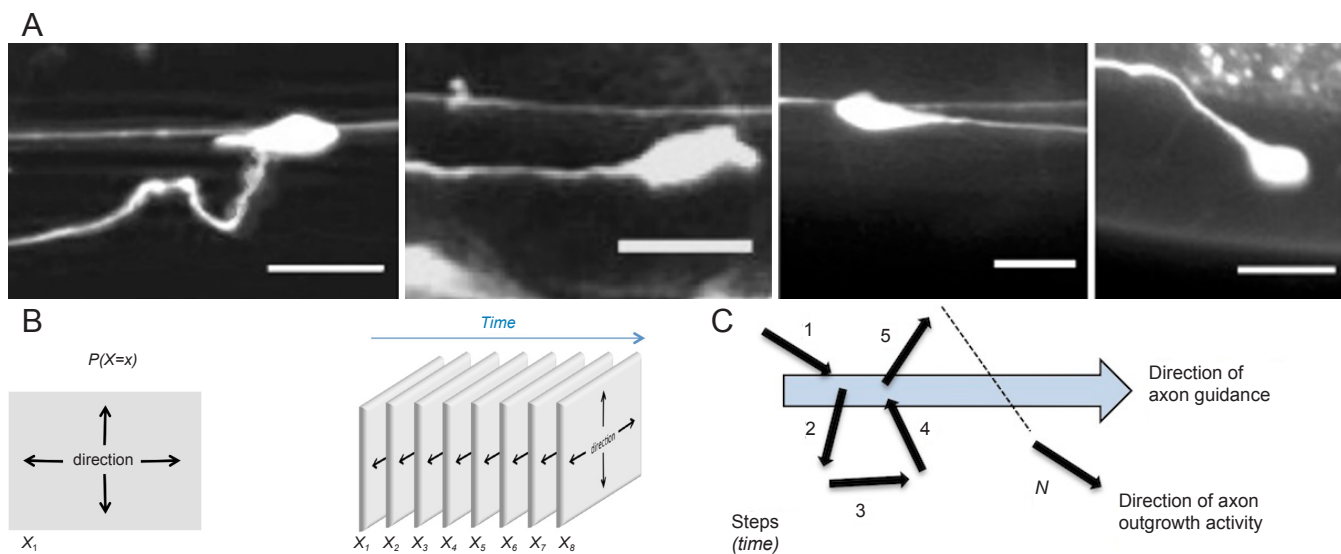


Figure 1 Describing axon guidance as a random walk.

(A) Photomicrographs showing the protrusion of the axon from the HSN cell body in different animals. In this mutant strain, the axon can extend either in the ventral (down), anterior (left), posterior (right), or dorsal (up) direction. Scale bars: 10 μ m.

(B) Illustration of a random walk. (left) At one discrete time, the probability distribution describes the probability of axon outgrowth activity occurring in a specific direction. (right) A random walk is a series of these steps over time. Direction (X) is a random variable that can take on a value (x) at each step.

(C) Illustration of a biased random walk. The direction of axon outgrowth is not necessary the same as the direction of guidance. Guidance cues induce or inhibit axon outgrowth activity at the molecular level. At each step (1, 2, 3, 4, 5... N) of the random walk the direction of outgrowth activity can differ. In aggregate these activities generate the observed outward movement of the axon.

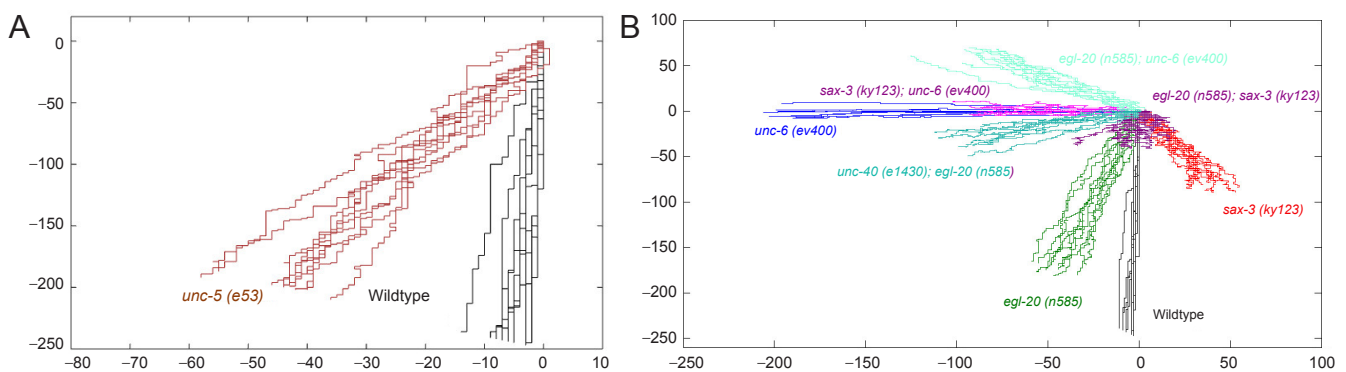


Figure 2 Random walks created from the probability of axon outgrowth from each side of the neuron.

(A) Visualization of the relative directional bias created by guidance cues in *unc-5* and wildtype animals. From measurements taken of the direction of axon outgrowth in *unc-5*(e53) or wild-type animals, the probability of dorsal, ventral, anterior, or posterior outgrowth was assigned to the direction of each step of a random walk moving up, down, left, or right, respectively. Each variable is considered independent and identically distributed. In each case, 10 simulations of 250 equal size steps were plotted based on a two-dimensional lattice random walk.

(B) Relative directional biases created by different combinations of mutations (Tang and Wadsworth, 2014). Shown are plots generated to compare the relative directional bias produced by different mutations that affect HSN axon guidance. 10 simulated random walks of 250 steps were plotted from an origin (0, 0). The walks were generated using the probabilities of outgrowth in the dorsal, ventral, anterior, and posterior direction. These are two-dimensional lattice random walks where each variable is considered independent and identically distributed.

alter the probability of outgrowth in another direction(s) as well. It further follows that the directional effect of a cue depends on the effects that all the other cues combined have on the probabilities.

We can observe this effect by comparing the relative directional bias created when different guidance cues are removed (Tang and Wadsworth, 2014). **Figure 2B** illustrates the directional bias created when the UNC-6 (netrin) or EGL-20 (wnt) cues are removed. Or when the UNC-40 (DCC) or SAX-3 (robo) receptors are removed. The directional bias in the *unc-6* mutant (blue), the *egl-20* mutant (green), and

the *egl-20; unc-6* double mutant (aqua marine) are each different. Therefore, the directional bias caused by the loss of UNC-6 requires EGL-20 and the directional bias caused by the loss of EGL-20 requires UNC-6.

The figure also shows that a cue can be required for guidance in different directions, depending on the presence of another cue or receptor. UNC-6 is required for the ventral (down) directional bias observed in wildtype, since when UNC-6 function is missing in the *unc-6* mutant (blue) the direction is anterior (left). But UNC-6 is also required for a posterior (right) directional bias when SAX-3 is missing

since there is a posterior directional bias in the *sax-3* mutant (red), but not in the *sax-3; unc-6* double mutant (magenta). Similarly, we can deduce from the single and double mutants that EGL-20 promotes the ventral bias in wild-type animals, the posterior bias in *sax-3* mutants, and the anterior bias in *unc-6* mutants.

Although I have discussed here the effects of secreted netrin and wnt guidance cues, it should be appreciated that once stochastically directed axon outgrowth activity is induced any interaction between the neuron and its environment that alters the probability of axon outgrowth at a side of the neuron effectively becomes a guidance cue.

Third, *guidance cues regulate the displacement of an axon*. Einstein was interested in describing how a Brownian particle would diffuse. By using the random walk approach he obtained the probability of a Brownian particle covering a particular distance in time *t*. He predicted that the displacement of the particle would not increase linearly, as a ballistic particle would, but instead would increase with the square root of time. Random walk movement has implications for understanding axon development. We observe that in mutants when the direction of axon outgrowth fluctuates, the development of a mature axon is delayed (Kulkarni et al., 2013; Tang and Wadsworth, 2014; Yang et al., 2014). This effect is also illustrated in **Figure 2B**. The average distance from the origin that the lines extend for *egl-20; sax-3* (purple) is much less than wildtype. Because of the random walk response, guidance cues are able to regulate the timing of axon maturation and the distance of axon outgrowth.

Finally, it is worth emphasizing that the “random walk” is a formal mathematical model. This model could be employed because it was recognized that the direction of axon outgrowth and UNC-40 localization from the cell body could be defined as a random variable and that a numerical value could be assigned for different possible outcomes (ventral, anterior, posterior, and dorsal) to create a probability distribution. Random walk guidance should not be equated with haphazard axon movement. Although “random” has also been used to describe changing patterns of axon outgrowth and UNC-40 intracellular localization, the “random” in this case refers to uncertainty and does not signify any well-defined stochastic process. Guidance by a random walk doesn’t necessarily mean that an axon will meander about. For example, the HSN axon does not wander in wild-type animals. Wandering movement may result through random walk guidance when there is greater variability in the direction of axon outgrowth activity, however more straight-line movement occurs as the probability of outgrowth activity in one direction approaches 1. In fact, the growth cones of some axons travel along well-defined tracts in response to contact-dependent cues. Interestingly, we have observed that a specific extracellular matrix, and the extracellular matrix protein UNC-52 (perlecan), provides direction information during UNC-40-mediated guidance by regulating the probability of axon outgrowth in different directions (Yang et al., 2014). In the context of random walk guidance, it is likely that many extracellular molecules that demarcate axon tracts play instructive guidance roles.

Conclusions: In this perceptive article, I have presented a probabilistic approach for understanding axon guidance. This approach may help reveal the practical effects of guidance cues *in vivo*. Although at the molecular level guidance cues might act as attractants and repellants for axon outgrowth they do not necessarily cause attraction or repulsion at the macroscopic level of axon guidance. This has implications for understanding nervous system development and the possible therapeutic use of guidance cues to treat diseases and injury.

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References

- Adler CE, Fetter RD, Bargmann CI (2006) UNC-6/Netrin induces neuronal asymmetry and defines the site of axon formation. *Nat Neurosci* 9:511-518.
- Einstein A (1905) On the movement of small particles suspended in stationary liquids required by the molecular-kinetic theory of heat. *Annalen der Physik* 17:549-560.
- Hedgecock EM, Culotti JG, Hall DH (1990) The *unc-5*, *unc-6*, and *unc-40* genes guide circumferential migrations of pioneer axons and mesodermal cells on the epidermis in *C. elegans*. *Neuron* 4:61-85.
- Ishii N, Wadsworth WG, Stern BD, Culotti JG, Hedgecock EM (1992) UNC-6, alaminin-related protein, guides cell and pioneer axon migrations in *C. elegans*. *Neuron* 9:873-881.
- Kennedy TE, Serafini T, de la Torre JR, Tessier-Lavigne M (1994) Netrins are diffusible chemotropic factors for commissural axons in the embryonic spinal cord. *Cell* 78:425-435.
- Kulkarni G, Xu Z, Mohamed AM, Li H, Tang X, Limerick G, Wadsworth WG (2013) Experimental evidence for UNC-6 (netrin) axon guidance by stochastic fluctuations of intracellular UNC-40 (DCC) outgrowth activity. *Biol Open* 2:1300-1312.
- Quinn CC, Pfeil DS, Chen E, Stovall EL, Harden MV, Gavin MK, Forrester WC, Ryder EF, Soto MC, Wadsworth WG (2006) UNC-6/netrin and SLT-1/slit guidance cues orient axon outgrowth mediated by MIG-10/RIAM/lamellipodin. *Curr Biol* 16:845-853.
- Ramón y Cajal S (1892) La Rétine des vertébrés. *La Cellule* 9:119-258.
- Tang X, Wadsworth WG (2014) SAX-3 (Robo) and UNC-40 (DCC) Regulate a directional bias for axon guidance in response to multiple extracellular cues. *PLoS One* 9:e110031.
- Wedlich-Soldner R, Li R (2003) Spontaneous cell polarization: undermining determinism. *Nat Cell Biol* 5:267-270.
- Xu Z, Li H, Wadsworth WG (2009) The roles of multiple UNC-40 (DCC) receptor-mediated signals in determining neuronal asymmetry induced by the UNC-6 (netrin) ligand. *Genetics* 183:941-949.
- Yang Y, Lee WS, Tang X, Wadsworth WG (2014) Extracellular matrix regulates UNC-6 (netrin) axon guidance by controlling the direction of intracellular UNC-40 (DCC) outgrowth activity. *PLoS One* 9:e97258.