Threshold mechanisms in acetylcholine pathway insecticides and environmental safety

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Overview

Previously we looked at some of the basic principles of nervous system function and how chemicals from several pesticide classes disrupt normal function. This time we will look in detail about what we can expect for a dose-response characterization of acetylcholine pathway insecticides based upon their mode of action and properties of the nervous system. This will get a little more technical than usual. The casual reader may want to skim over the equations but think about the explanations.

Several families of pesticides function by disrupting the synaptic acetylcholine pathway. Organophosphate pesticides and the carbamates block the enzyme acetylcholinesterase (**AChE**) such that the naturally released neurotransmitter, acetylcholine (**ACh**), is not broken down and recycled. Instead **ACh** builds up in the synaptic junction and over-stimulates the acetylcholine receptors (**AChR**) on the post-synaptic membrane.

The neonicotinoids act directly by bonding strongly to the nicotinic acetylcholine receptors (**nAChR**) in a manner that holds open the receptor ion channel.

Both classes of chemicals, the **AChE** inhibitors and the **nAChR** agonists, produce excessive numbers of activated acetylcholine receptors on the post synaptic membrane, which gives rise to a reduction in the post synaptic resting potential and a propensity to generate action potentials in the post synaptic neuron. Acute poisoning occurs when the general level of neural stimulation is sufficient to disrupt the normal physiological processes required to sustain life. Clinically, insects and animals poisoned with either class of chemicals are seen to lose muscular control, exhibit uncontrolled twitching, eventual paralysis, and death.

We will begin by considering a single synapse and come up with a relationship for the post synaptic stimulation as a function of the fractional lethal chemical level. We will also consider implications of acetylcholinesterase disruption for an entire neural network. Finally we will seek to understand the environmental implications of threshold versus non-threshold action with these chemicals.

Synaptic electro-chemical function

The nervous system is governed by neuronal generated “action potentials”, rapid electrical potential changes that travel rapidly along the neural axons and terminate in the branching tree of

dendrites at synapses where they can cause the release of neurotransmitter into the synaptic cleft. The neurotransmitters rapidly diffuse across the synaptic junction and attach to receptors on the post-synaptic membrane. These transiently bound receptors change the permeability of the membrane and allow ion currents to flow across the membrane, thus altering the local cellular electrical potential in the post-synaptic neuron.

Action potentials are fast transients that last 1-3 milliseconds. Diffusion time of **ACh** across the junction is faster, measured in microseconds, and the decay of synaptic free-circulating **ACh** is normally around one millisecond. The time response of the excitatory post-synaptic potential is slightly slower, typically lasting several to 10's of milliseconds.(1) This allows the post-synaptic neuron to be the summing junction from many synaptic inputs, doing some kind of dynamic averaging that determines whether or not the downstream neuron will produce its own action potential. We argue that changing the decay time of **ACh** in the synaptic junction relates directly to stimulation that will produce an action potential in the downstream neuron. Roughly, doubling the decay time is likely to double the likelihood of the downstream neuron generating its own action potential because the amount of post-synaptic charge transfer will be proportional to the length of time **ACh** holds open the **AChR** receptors, and this open time is within the typical averaging period of the neuron.

Acetylcholinesterase Inhibitors - Consider a single synapse

A normal stimulus, S_0, produced by a single action potential in the downstream neuron can be written as

[1] $$S_0 = kN_R N_A \tau_A$$

Where N_R is the concentration of **AChR**s, N_A is the concentration of **ACh** released by the action potential, k is a proportionality constant and τ_A is the lifetime of **ACh** in the synaptic junction.

The primary way acetylcholinesterase inhibitors act is by reducing the number of **AChE** molecules available to catalyze the destruction of **ACh** in the synaptic junction. It is reasonable to expect that decreasing the number of available **AChE** molecules will proportionally increase the time it takes for **ACh** molecules to be degraded. If we assume that a fraction, f, of the **AChE** is bound with inhibitor, then we estimate the **ACh** lifetime, τ, in the presence of **AChE** inhibitor as

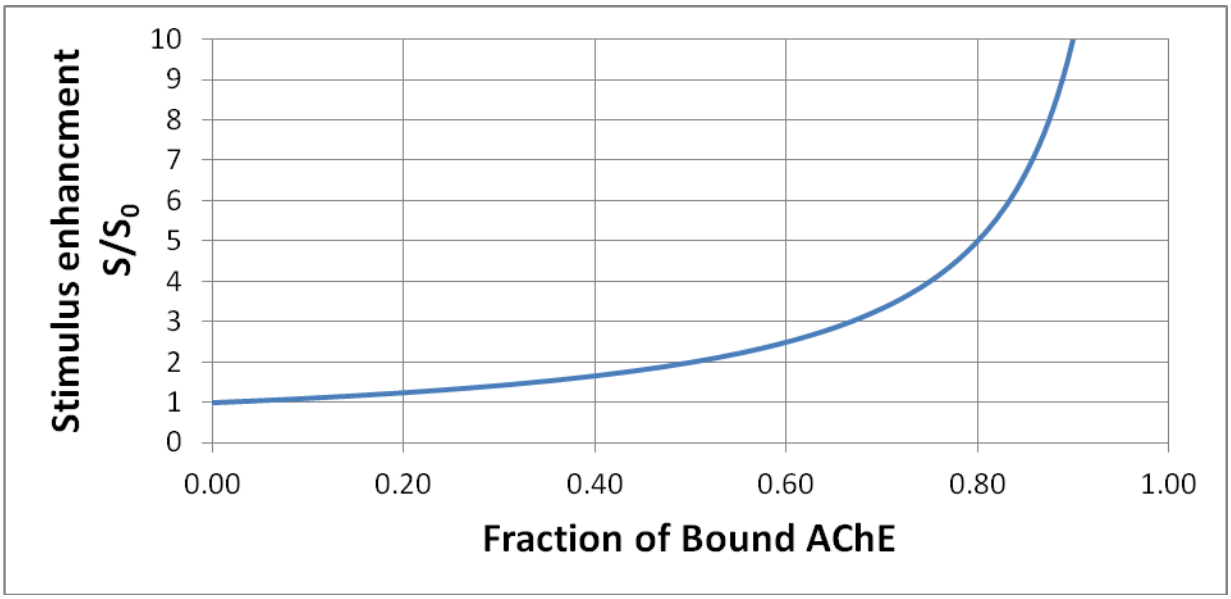
[2] $$\tau = \tau_A / (1 - f)$$

For a single action potential neither N_R nor N_A are affected by the **AChE** inhibitor, so we can express the excess stimulus as a function of the fraction of inhibited **AChE** as

[3] $$S = S_0 \left(1 - \frac{1}{(1-f)}\right)$$

As f increases, eventually the excess stimulus is lethal which we designate as S_L, occurring at f_L.

[4] $$S_L = S_0\left(1 - \frac{1}{(1-f_L)}\right)$$



The graph shows what happens as the fraction of bound **AChE** increases. The stimulus enhancement rapidly gets large as most of the **AChE** becomes unavailable to catalyze the destruction of **ACh**.

With a little algebra you can show that the fraction of excess stimulation at the sub lethal limit compared to lethal over-stimulation can be expressed as

[5] $$\frac{S_\epsilon}{S_L} = \epsilon\left(1 - f_L\right)$$

Where $\epsilon = f/f_L << 1$ is the sub-lethal exposure as a fraction of the lethal level, and S_ϵ is the excess simulation associated with the small dose ϵ.

Example: If the lethal stimulus level is five times the normal background level of neuronal activity, then 80% of the **AChE** must be bound. If we ask what happens with an exposure that is 10% of the lethal level, (8% of **AChE** bound) then the increase in simulation is only 1.6% of the increase needed for lethality. In the residual limit, the stimulus increase is less-than-linear with exposure, with this "safe residual" effect strongest for chemicals where f_L approaches 1.

Studies with fish have shown that **AChE** inhibition levels need to be 60% to 90% (2), depending upon the chemical and species, to be lethal. This is more or less in accord with this model

where lethality requires most of the **AChE** receptors to be out of commission, and would suggest that toxicity suppression for residual levels would be significant for these chemicals.

Acetylcholine Dynamics - Consider the complete network

Now we will take the nervous system as an ensemble of neurons with average properties. Specifically we are interested in the acetylcholine pathway, so we define several global average quantities and relationships between them. Acetylcholine activates receptor sites on the post synaptic membrane that stimulate the post synaptic neuron. We can express this globally averaged stimulus, S_{ACh}, as

[6] $$S_{ACh} = kN_{ACh}N_R$$

where k is a proportionality constant, N_{ACh} is the average concentration of synaptic acetylcholine, and N_R is the concentration of acetylcholine receptor sites. (Unlike the previous section, here N_{ACh} is an averaged network concentration, whereas N_A in Equation [1] described the total release of **ACh** caused by a typical action potential.) Acetylcholine is release into the synaptic junction by action potentials from stimulated neurons. It is then quickly degraded by acetylcholinesterase receptors located in the synaptic cleft. We can express this relationship as

[7] $$\frac{dN_{ACh}}{dt} = k'S_{ACh} - k_E N_{ACh}N_E$$

Where k' reflects the efficiency of the averaged stimulus at generating additional **ACh** due to stimulus-induced action potentials. N_E is the concentration of **AChE** that degrades **ACh** and k_E is a constant involving the efficiency for **AChE** destruction of **ACh**. Combining [6] and [7] and defining $kk' \equiv k_R$, we get

[8] $$\frac{dN_{ACh}}{dt} = \left(k_R N_R - k_E N_E\right) N_{ACh}$$

A solution of the differential equation is

[9] $$N_{ACh} = N_{ACh0}e^{t/\tau}$$

Where the growth rate, τ is

[10] $$\tau = 1/\left(k_R N_R - k_E N_E\right)$$

The growth rate, τ, must be negative or acetylcholine concentration will grow without bounds,

[11] $$N_E > \frac{k_R}{k_E} N_R$$

Hence, under normal conditions, the concentration of **AChE** must be sufficient to prevent runaway growth of the **ACh** concentration due to **ACh**'s ability to generally stimulate the neural network. Here we are not considering the many other neurotransmitters, both agonists and inhibitors, that are included in the network, nor are we considering external inputs. However, conditions that place the entire network in a rough dynamic balance enhance the network's ability to involve multiple neurons for information processing. Hence, one might suspect that the inequality [11] is only weakly maintained, at least in some portions of the neural network since this would lead to a network that would be more optimal for information processing .

The complete network with AChE Inhibitors

Now let us consider what happens when we add **AChE** inhibitors and to this picture. The effect of chemical **AChE** inhibition will be to reduce the natural concentration **AChE**, N_{E0}, to an available active component

[12] $$N_E = N_{E0}(1 - f)$$

where f is the fraction of bound **AChE** receptors. Substituting [12] into [11] and solving for f, we find that there is a critical inhibition fraction that will result in uncontrolled growth of the ACh concentration.

[13] $$f_{Crit} = 1 - \frac{k_R N_R}{k_E N_{E0}}$$

One might consider this the threshold level at which **AChE** pesticides produce a lethal effect.

In the medical literature on OP poisoning, one comes across the notion of "cholinergic crisis" which suggests such a threshold-like condition (3). Although experimentally it is found that relatively large fractions of the **AChE** must be inhibited to cause lethality, this network effect may play the role of the coup de grâce at the entire organism level.

Acetylcholine Receptor Agonists - Neonicotinoids

The acetylcholine receptor agonists such as the neonicotinoids will directly stimulate the post synaptic neuron. We can write the postsynaptic stimulation, S_{Nic}, due to the neonicotinoid as

[14] $$S_{Nic} = j_C f N_R$$

Where j_C is the single-receptor ion current stimulation, N_R is the **nAChR** concentration and f is the fraction of receptors bound with agonist. When only a few receptors are bound with agonist, the cell's ion pumps will attempt to restore the resting potential of the neuron. However, ion pumps are a slow energy-intensive process. Compared to an open **nAChR** channel, as a rough estimate, an ion pump will only generate ~10^{-5} as much current.(4) Put another way, for each open **nAChR** there needs to be ~ 10^5 ion pump channels in action to keep the cell in homeostasis. A normal functioning **nAChR** would remain activated only for a few milliseconds

at most, so much less pumping is required to recover from normal activity because of the low synaptic duty cycle.

No obvious threshold mechanisms are present for this class of chemical. Instead, the excess stimulation is directly proportional to the amount of bound receptors, which is itself proportional to insecticide dose. If we go through the exercise like we did for equation [5] we discover that in the residual limit where $\epsilon << 1$,

[15] $$\frac{S_\epsilon}{S_L} = \epsilon$$

showing the stimulus is proportional to the residual dose.

Metabolic load for residual levels of these chemicals

Any depolarization of the post-synaptic neuron must be eventually be rectified by metabolic processes that pump ions uphill against the gradient to return the neuron to its normal resting potential. Chemicals that increase the post-synaptic stimulation beyond the natural level will require proportionately more metabolic effort to return the neuron to its resting potential. For the **AChE** inhibitors the excess stimulation is only operative when the synapse is stimulated by the action potential and **ACh** is present. If we wish to find an averaged excess stimulation of the post synaptic neuron, we need to multiply the instantaneous excess stimulation by the synaptic duty cycle, D. We can rewrite equation [5] for the averaged excess stimulation for a residual quantity of **AChE** inhibitor, ϵ_{OP} as

[16] $$\langle S_{\epsilon_{OP}} \rangle = \epsilon_{OP} \left(1 - f_L\right) D S_{L_{OP}}$$

For the neonicotinoids, the stimulation is constant, with duty cycle equal to one when doing the time averaging.

[17] $$\langle S_{\epsilon_{NN}} \rangle = \epsilon_{NN} S_{L_{NN}}$$

If we assume that chemical pesticides are applied in the field at rates that are designed to produce a lethal effect in target organisms, then we can compare the relative effects of residual levels of the chemicals $\epsilon = \epsilon_{OP} = \epsilon_{NN}$, some small fraction of the lethal level, by normalizing to an application rate where $S_{L_{OP}} = S_{L_{NN}}$. Here the subscripts OP and NN refer to the organophosphate or neonicotinoid classes of chemicals respectively. With these assumptions, combining [16] and [17],

[18] $$\frac{\langle S_{\epsilon_{OP}} \rangle}{\langle S_{\epsilon_{NN}} \rangle} = \left(1 - f_L\right) D$$

The above comparison suggests that for similar residual levels of the two classes of chemicals, the neonicotinoids will produce a much larger average post-synaptic stimulation. We can make estimates for the synaptic duty cycle based upon observed average firing frequency, ~1 Hz, and

typical action potential duration, ~2 ms. If we assume the threshold term $(1 - f_L) = 0.5$, then taken together the neonicotinoid chemicals will produce ~1000 times more averaged post-synaptic stimulation than would similar residual levels of organophosphate pesticides. For sub lethal doses of the pesticides where nervous system function is not strongly impaired, the primary physiological effect one would expect to see would be a much higher metabolic drag on the organisms exposed to low levels of neonicotinoids.

Time Cumulative Effects

The time history of the the movement of the pesticide from its initial application, its interaction with target or non-target organisms, and its eventual dilution and degradation can have dramatic consequences in terms of both acute initial toxic effect and latent residual toxic effect.(5) An effective and safe pesticide should strongly attack the target organism yet remain benign to similar species that are *not* the target organisms. The best way to achieve a strong differentiation from initial application compared to residual pollutant is to use chemicals that have all of the following properties:

1. Rapidly degrade in the environment.
2. Rapidly disassociate at targeted biological binding sites.
3. Have a strong threshold action.

Let us look at these in turn. Persistent chemical pollutants have been the bane of the pesticide industry since DDT. None of the acetylcholine path insecticides are as bad as the organochlorines, but there is still quite a difference between members of this group. The neonicotinoids are said to have around a 1 year soil life, but experience suggests that to be an optimistic number. Where the chemicals have been used for many years, the contamination levels continue to increase. Since the neonicotinoids are water soluble, this suggests that what may appear as degradation is merely dilution and migration. Instead of the chemical disappearing, we find contamination far from the source of the application. (6,7,8) Chemicals that are persistent in the environment long after the crop is harvested and target insects are gone can only have deleterious consequences for unintended organisms. The severity of the consequences depends on the final two properties.

Insecticide chemicals that bind to targeted receptors can have a wide range of receptor affinity and binding strength. Chemicals that bind transiently (like the **ACh** molecule itself to **AChR**s) will remain in quasi chemical equilibrium with the extracellular fluid and will bind to target molecules at a rate that is proportional to the concentration of the chemical. However, some insecticide chemicals are designed to bind tenaciously to the desired receptor sites. In these cases, the molecules will become trapped at the target site even after most of the chemical has been rid from the organism's body by metabolic processes. In cases with very strong target binding, one can expect accumulation over time of molecules at the target sites as long as there is any continuing exposure to the chemical. How serious a problem this will be for non-target organisms depends on the last property, whether the chemical works with a threshold action or not.

Properties of Pesticide Classes

Chemical neuro-toxic pesticides have been widely used for more than 70 years. During that time several families of chemicals have been developed to target specific neurological receptors. The chart below lists several of these classes, includes a common example or two from each class and shows typical properties of these chemicals.

Pesticide Class	Example Chemical	Oral LD50 Honey-bees	Typical Soil half-life	Typical metabolic half-life	Typical binding dissocia-tion time	Typ. tox. time-scaling exponent	Toxic Mechan-ism	Comment
Neonic-otinoids	Imidacloprid	50 ng/bee	.5 – 3 yr.	4 hr.	>10 days	2	Synaptic nAChR agonist.	Often used as systemic insecticides
	Thiameth-oxam	20 ng/bee	30-300 days	2-6 hr. (rats)	?	2	Irreversible binding	Direct acting on nAChRs
Pyrethroids	Delta-methrin	60 ng/bee	11-72 days	2 hr.	Several seconds	2 ?	Keeps open voltage gated Na+ ion channels on axon	Direct acting on Na+ channels
Organo-chlorines	DDT	6190 ng/bee	2-15 yr.	6 yr.	Temperture dependant-- suggests less than a second.	?	Keeps open voltage gated Na+ ion channels on axon	Most of these chemicals have been banned by international treaty as persistent organic pollutants
	Dieldrin	133 ng/bee	5 yr.	9-12 mo. humans		?		
Organo-phosphate	Diazinon	370 ng/bee	15-200 days	17 hr.	16 days	1 ?	Irreversible AChE inhibitor	AChE inhibitors have inherent "threshold" action. A large fraction of AChE must be bound to have toxic effect Indirect acting on ACh
	Malathion	720 ng/bee	1-15 days	12 hr.	? days	0.5 (fish)		
Carbamates	Carbaryl (Sevin)	1540 ng/bee	4-30 days	8 hr.	short	1	Reversible AChE inhibitor	

It is worth looking at the typical chemicals in the table above in light of the requirements we identified as desirable for a safe pesticide. Note that the organochlorines failed badly because they were so persistent in the environment to the point they have been almost universally banned. They were largely replaced by the organophosphates with which we've continue to have an uneasy coexistence for the last half-century. Under scrutiny because of their potent effects on humans and other vertebrates, many of the organophosphate insecticides are being forced into retirement. The replacement has been the neonicotinoids, which have the benefit of relative specificity to invertebrate **nAChR** receptors making the chemicals less toxic to humans and other vertebrates. Unfortunately, the neonicotinoids fail with regard to all three of the properties for safe and effective pesticides.

From our discussion you can see that the safest chemicals are the carbamates. Typically it takes more chemical (compare LD50 for neonicotinoids, organophosphates, and carbamates) to kill the target insect, but the persistence of the chemical in the environment is short. It is metabolized relatively quickly, and acts reversibly with the target receptors. Finally, it is also an **AChE** inhibitor that has a strong threshold of action effect. Compare this with the neonicotinoids at the top of the chart. It takes much less neonicotinoid chemical to kill, but this is likely due to its tenacious persistence on the target receptor sites. The chemicals do not degrade very quickly in the environment so they will continue to accumulate on target and non-target organism synaptic receptors long after the initial application. And finally, the neonicotinoids produce toxic effects at residual dose levels, unlike the **AChE** inhibitors. All of the tricks we have in the playbook to segregate between target and beneficial insects fail with the neonicotinoids.

Implications of threshold action for toxicity scaling

Change of sign of the acetylcholine growth rate provides a clear qualitative turning point for the organism. It is easy to understand how such a runaway event can lead to death. Hence, if you wish to model the toxicity scaling of a compound with such a distinct threshold action, all you have to do is follow the movement of toxin to receptor sites until the threshold is reached. This will naturally give you Haber's rule for substances that accumulate, such as most of the organophosphate insecticides. For insecticides that don't accumulate on receptors, such as carbamates, one would expect threshold action without significant time dependence. Once pesticide concentrations reached levels where chemical equilibrium at receptor sites resulted in enough bound **AChE** to change the sign of the **ACh** growth rate, the threshold condition would be reached. However, at small residual concentrations of acetylcholinesterase inhibitors, the molecules disable a few AChE sites and hence slightly change the synaptic response, but otherwise remain largely benign to the organism. For this class molecule, there is a very large change in toxic effect with concentration. Despite the continued environmental issues and concerns with organophosphate pesticides, it should be recognized that they may be intrinsically environmentally safer because of their strong threshold action than the newer neonicotinoids.

For the neonicotinoids where there is no distinct threshold condition, the situation is more complicated. The transition from alive to dead is not accompanied by a convenient mathematical marker like the change in sign of a growth rate. Especially at the residual limit, we are left to speculate on the physiological impact of accumulate insults from the toxic chemical. Single molecules will open ion channels and begin to depolarize neurons. This abnormal state of affairs would be countered by energy-burning processes in the organism to mitigate the dysfunction. This is the definition of stress. It is likely that the residual-level stresses to non-target organisms is the Achilles' heal for the neonicotinoid insecticides. Very low concentrations of these pesticides have the potential to switch on compensatory physiological processes that are poorly understood, but likely stressful. One example was the discovery that very low levels of the neonicotinoid clothianidin reduced the immune response of honeybees to the point where deformed wing virus could replicate. Low levels of the acetylcholinesterase inhibitor chlorpyriphos, the molecules of which in our understanding would be rather benignly latched on to a few of the **AChE** sites, showed no such immune suppression effect.(9) The fact that **nAChR** channels are involved in less well studied immune system and cellular signaling

functions adds to the risk that disrupting these pathways will have unintended consequences.(10,11)

A key point is that at residual levels, **AChE** inhibitors are really doing nothing. A small fraction of the **AChE** sites may be out of commission, but even that effect is only apparent when the neuron fires and there is **ACh** to be swept away. During the neuron's quiet state the pesticide molecules are benign. Contrast this situation with what happens on the postsynaptic membrane with a few neonicotinoid molecules. Single neonicotinoid molecules hold open **nAChR** channels that will tend to depolarize the neuron. This happens even when the neuron is in an un-stimulated state. However, given the persistent depolarization by the open channel, it can't rest. Instead the cell must muster energetic processes in an attempt to restore the neuron's polarization so that it may still function.

Besides suppression of immune response as mentioned above, there are likely other detrimental effects from the energy sapping response required by residual neonicotinoid poisoning. Trade-offs between energy expenditures to maintain neurological function and more normal activities such as powering flight muscles may explain some of the observed effects of chronic low level exposure. (12) Another study shows epi-genetic changes to imidacloprid-exposed honeybee larva that that strongly affects genes involving metabolism. (13) The myriad effects that low level neonicotinoid exposure presents, such as impaired navigation, poor learning ability, reduced flight time, and immunological impairment may be better understood from the perspective of the metabolic stress caused by open nAChR channels than by direct neurological impairment.

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