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Binary Mixtures of Pyrethroids Interact with Voltage-Sensitive Calcium and Chloride Channels in Isolated Presynaptic Nerve Terminals from Rat Brain

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BINARY MIXTURES OF PYRETHROIDS INTERACT WITH VOLTAGE-SENSITIVE CALCIUM AND CHLORIDE CHANNELS IN ISOLATED PRESYNAPTIC NERVE TERMINALS FROM RAT BRAIN

A Thesis Presented

by

HILLIARY E. HODGDON

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DEDICATION

To my family and friends for their guidance and support.

ACKNOWLEDGMENTS

ABSTRACT

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SEPTEMBER 2008

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Select pyrethroid binary mixtures (deltamethrin plus *S*-bioallethrin, β-cyfluthrin, cypermethrin, and fenpropathrin) elicit a more-than-additive response on L-glutamate release from rat brain synaptosomes that is independent of calcium influx. Using a variety of chloride channel antagonists, anthracene-9-carboxylic acid (9-AC), rChlorotoxin (ClTx), 4,4'-dintitrostilbene-2,2'-disulfonic acid (DNDS), 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB), and picrotoxinin (PTX), we have identified two mechanisms by which pyrethroids may enhance L-glutamate release. The results from this study indicate that only ClTx and NPPB, at their EC_{50}s (0.1 μM and 70 μM, respectively), significantly increase L-glutamate release when in the presence of our most potent pyrethroid, deltamethrin, at its EC_{50} (2×10^{-12} M). When these two antagonists were used in the presence of deltamethrin plus cypermethrin and deltamethrin plus fenpropathrin, a more-than-additive response was elicited at lower concentrations of the binary mixtures. Likewise, NPPB in the presence of the additive binary mixture, deltamethrin plus tefluthrin, first elicited a more-than-additive response at the 1:10

mixture. Since both ClTx and NPPB are inhibitors of voltage-gated chloride channels (ClC-2) and calcium-activated chloride channels, our findings suggest that these channels are potential target sites for certain pyrethroids and likely are important in pyrethroid neurotoxicity.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

1.1 Regulatory History of Pyrethroids

The United States Environmental Protection Agency (USEPA) regulates pesticides under two major statutes: the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) and the Federal Food, Drug, and Cosmetic Act (FFDCA). Under FIFRA, the EPA is responsible for registering pesticides for use in the United States and prescribing regulatory requirements to prevent unreasonable adverse effects on human health and the environment while the FFDCA requires the EPA to establish tolerances for pesticide residues in food (United States Environmental Protection Agency 2007c). In 1996, Congress passed the Food Quality Protection Act (FQPA) that mandated the USEPA to complete the review and reassessment of the tolerances (maximum legally permissible levels) for all food use pesticides within a decade (United States Environmental Protection Agency 2007c).

Since the enactment of the FQPA, many efforts have been made to update and resolve inconsistencies between the two major pesticide statutes by establishing a more consistent and protective regulatory scheme including, a mandate of a single, health-based standard for all pesticides in all foods, an expansion of the scope of protection for infants and children, and a reassessment of the safety of existing tolerances and tolerance exemptions (United States Environmental Protection Agency 2007a).

N-methyl carbamates and organophosphates are among the classes of pesticides being reassessed under the FQPA for cumulative risk since some share a common mechanism of toxicity (e.g., act the same way to produce toxicity).   In conducting these cumulative risk assessments, the EPA must evaluate the potential for a person to be exposed to more than one compound from food, drinking water, and residential sources (United States Environmental Protection Agency 2007b).

Thus far, findings from the cumulative risk assessments have led to the discontinued or restricted use of certain pesticides from the carbamate and organophosphate classes of insecticides (United States Environmental Protection Agency 2007a, United States Environmental Protection Agency 2006). It is expected that the use of other control agents, specifically the pyrethroid insecticides, will increase in order to ensure and maintain agricultural productivity and control of insect vectors of human and animal diseases.

Pyrethroids account for approximately one quarter of the worldwide insecticide market and have been used since the 1970s as control agents in urban areas and to control insects that vector diseases such as malaria, dengue fever, and West Nile virus (Casida, J.E. 1998; Narahashi, T. 1992; Trainer, V.L. 1997; Takken, W. 2002). Pyrethroids are synthetic insecticides that were optimized based on the structures of the six naturally-occurring pyrethrin esters of the pyrethrum extract derived from the flower heads of *Chrysanthemum cinerariaefolium* (Soderlund, D.M. 2002). For almost two centuries, pyrethrum was the most important botanical insecticide because of its potency and selectivity to insects. Pyrethrum, however, is highly susceptible to hydrolytic ester cleavage and is unstable in light and air, greatly diminishing its effectiveness as a field

stable insecticide (Casida, J.E. 1998; Soderlund, D.M. 2002). The development of synthetic pyrethroids was the result of efforts to increase their photostability and acute neurotoxicity in insects by altering either the cyclopropanecarboxylic acid or cyclopentenolone alcohol moieties of the pyrethrin esters (Fig. 1) (Soderlund, D.M. 2002). In fact, mammals are three orders of magnitude less sensitive to the toxic action of pyrethroids than insects owing to their rapid metabolism, poor absorption, higher body temperature (pyrethroids have a negative temperature coefficient of action), and lower sensitivity of ion channel target sites (Clark, J.M. 1994; Narahashi, T. 1992; Soderlund, D.M. 1989; Ray, D.E. 2006). Given the high risk of exposure due to the widespread use of pyrethroids in agriculture and public health and the potential for their usage to increase, the EPA must likewise consider the cumulative risk of pyrethroids in mammals under the FQPA criteria.
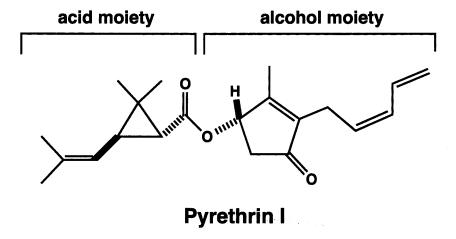
Figure 1. The chemical structure of pyrethrin I, the most toxic ester of pyrethrum. This structure is the basis for all pyrethroids (reproduced from Soderlund 2002).

Overall, the entire molecular shape and stereospecificity of pyrethroids rather than a specific substructure, reactive entity, or molecular moiety of the compound, are responsible for pyrethroid toxicity (Elliott, M. *et al.* 1974; Soderlund, D.M. 200). The acute neurotoxicity of pyrethroids has been well characterized and an initial classification based on their symptomology in rats has been established (Verschoyle, R.D. 1980; Shafer, T.J. 2005). Pyrethroids producing hypersensitivity, aggression, convulsions, and fine tremor progressing to whole-body tremor were termed tremor (T) syndrome. Pyrethroids producing pawing and burrowing, salivation, and coarse tremor progressing to choreoathetosis (sinuous writhing convulsions) were termed choreoathetosis with salivation (CS) syndrome (Verschoyle, R.D. and Barnes, J.M. 1972; Lawrence, L.J. 1982; Gammon, D.W. 1981). An alternative nomenclature for pyrethroids was later proposed based on their chemical structures and their toxicological effects produced in insects. Pyrethroids containing an α-cyano-3-phenoxybenzyl alcohol group were called Type II pyrethroids whereas compounds lacking an α-cyano group were called Type I pyrethroids (Lawrence, L.J. 1982; Gammon, D.W. 1981). Although the Type I/II nomenclature has been widely adopted in literature and is often used synonymously with the T/CS syndrome terminology respectively, some pyrethroids exhibit an intermediate syndrome composed of both T and CS syndromes, some Type I pyrethroids elicit the CS-syndrome (permethrin), and some Type II pyrethroids elicit the T-syndrome (fenpropathrin) (Verschoyle, R.D. 1980; Lawrence, L.J. 1982; Soderlund, D.M. 2002).

In the 1970s, Aldridge *et al.* demonstrated that the *in vivo* action of deltamethrin (CS-syndrome pyrethroid) on the nervous system was different from that of cismethrin (T-syndrome pyrethroid) and DDT. After treating rats orally with DDT, cismethrin, and

deltamethrin, they found that deltamethrin caused a 52% decrease in acetylcholine content of the brain and cerebellum whereas DDT and cismethrin caused no significant reduction in the amount of neurotransmitter present (Aldridge, W.N. 1978). A more recent study conducted on freely moving rats validated this *in vivo* finding and showed that deltamethrin caused a concentration-dependent efflux of acetylcholine at the onset of the CS symptomology (Hossain, M.M. 2004). Collectively, these findings suggested that CS-syndrome pyrethroids act differently at the presynaptic nerve terminal than T-syndrome pyrethroids. This difference may be responsible for the different neurotoxic action of CS-syndrome pyrethroids versus T-syndrome pyrethroids (Ray, D.E. 1979).

Over the past three decades, voltage-gated sodium channels (VGSC) in nerve axons have been well documented as a principal site of action of all pyrethroids in both insects and mammals (Soderlund, D.M. 1989; Narahashi, T. 1992; Soderlund, D.M. 2000). Intracellular recordings of action potentials in nerve axons demonstrated that pyrethroids produce two distinct types of effects on nerve excitability depending on the structure of the pyrethroid. Type I poisoning is characterized by a slowing of VGSC inactivation, resulting in a depolarizing after potential (DAP) due to an increase in "late current". The DAP results in the spontaneous firing in action potentials and ultimately long trains of repetitive discharges, which are directly correlated to the tremors produced by T-syndrome pyrethroids and are qualitatively similar, if not identical, to the actions of the well established VGSC agonist, DDT (Narahashi, T. 1960; Lund, A.E. 1983; Soderlund, D.M. 2000; Narahashi, T. 1992; Ray, D.E. 2006). Type II pyrethroids that produce the CS-syndrome most noticeably slow the deactivation kinetics of VGSCs,

resulting in a prolonged sodium tail current that eventually causes conduction block (Shafer, T.J. 2005; Soderlund, D.M. 2002).

In addition to VGSCs, ATPases, L-glutamate receptors, acetylcholine receptors, gamma aminobutyric acid (GABA) gated chloride channels, voltage-gated calcium channels (VGCCs) and chloride channels (VGClCs) have been identified as potential molecular targets of pyrethroid activity (Soderlund, D.M. 2002). Only the latter two (VGCCs and VGClCs), however, are altered by relatively low concentrations of pyrethroids, elicit stereospecific actions, and have been implicated in the acute neurotoxicological response in functional assays (Hildebrand, M.E. 2004; Ray, D.E. 2000; Soderlund, D.M. 2002; Symington, S.B. 2007b).

Evidence for VGCCs as a site of action for pyrethroids at the presynaptic nerve terminal has been demonstrated using isolated presynaptic nerve terminals (synaptosomes) from rat brain (Symington, S.B. 2007b). Upon depolarization, both cismethrin, a T-syndrome pyrethroid, and 1R-deltamethrin, a CS-syndrome pyrethroid, resulted in a concentration-dependent increase in calcium influx whereas their inactive stereoisomers, bioresmethrin and 1S-deltamethrin respectively, did not (Symington, S.B. 2007b). Furthermore, though both cismethrin and 1R-deltamethrin stimulated calcium influx, only 1R-deltamethrin enhanced Ca^{2+}-dependent neurotransmitter (L-glutamate) release. L-glutamate release was stimulated by K^+ depolarization, unaltered by the VGSC antagonist, tetrodotoxin, and blocked by ω-conotoxin GVIA, the N-type calcium channel antagonist, implicating the N-type VGCCs as target sites for CS-syndrome pyrethroids (Clark, J.M. 2007).

The stereospecific action of pyrethroids was further validated using heterologously expressed $Ca_V2.2$ (N-type calcium channel from rat brain) in *Xenopus laevis* oocytes. Contrary to their findings in synaptosomes, Clark found that 1R-deltamethrin reduced peak current in a concentration-dependent manner via wild-type channels (T422) while the inactive stereoisomer, 1S-deltamethrin, had no effect. In a permanently phosphorylated form of $Ca_V2.2$ (T422E mutant), however, peak current increased in the presence of 1R-deltamethrin while 1S-deltamethrin had no significant effect (Clark, J.M. 2007). The permanently unphosphorylated form of $Ca_V2.2$ (T422A), unlike the wild-type and T422E channels, displayed a significant decrease in peak current in the presence of both the deltamethrin stereoisomers (Symington, S.B. 2007a). Since 1R-deltamethrin was ~20-fold more potent on T422E $Ca_V2.2$ compared to wild-type, T422 $Ca_V2.2$, the permanently phosphorylated form of $Ca_V2.2$ is assumed to be the preferred target of deltamethrin. This contention is further supported by the observation that protein phosphorylation patterns in synaptosomal preparations are modified by deltamethrin, possibly by a Ca^{2+}/diacylglycerol-dependent protein kinase (PKC)-dependent pathway (PKC is the protein kinase that phosphorylates the T422 residue in $Ca_V2.2$) (Matsumura, F. 1989; Zamponi, G.W. 1997; Enan, E. 1991; Clark, J.M 2008). Thus, the differences in the action of deltamethrin on the functional synaptosomal preparations versus the heterologously expressed $Ca_V2.2$ suggest that other regulatory components modulating Ca^{2+} influx, including channel phosphorylation, may be necessary to elicit an *in vivo* neurotoxic response (Symington, S.B. 2007a; Catterall, W.A. 1997; Catterall, W.A. 1998).

With respect to pyrethroids presenting a cumulative risk, it was shown that certain binary mixtures of pyrethroids at their respective EC_{50}s (equipotent binary mixtures) resulted in a more-than-additive effect on neurotransmitter release that was not Ca^{2+} dependent (did not further modify VGCCs) (Table 1) (Frisbie, R.K. 2006). These mixtures included deltamethrin in binary combinations with *S*-bioallethrin, β-cyfluthrin, cypermethrin, or fenpropathrin. This subgroup of pyrethroids does not fit cleanly into either the neurotoxic syndrome (T- or CS- syndrome) or structural (Type I or II) classifications. For example, fenpropathrin, a type II pyrethroid, elicits the T-syndrome. This subset of pyrethroids, however, has been identified as calcium-independent VGClC antagonists¸ implicating the importance of possible modification of VGClCs in the neurotoxicological response of pyrethroids in mammals (Burr, S.A. 2004).

Originally, Forshaw *et al.* suggested that VGClCs were target sites for certain pyrethroids using patch clamp electrophysiology. In mouse N1E-115 neuroblastoma cells, low concentrations of 1R-deltamethrin, but not cismethrin, decreased the open channel probability of voltage-gated maxi chloride channels. The deltamethrin effect could be abolished with the addition of the chloride channel agonist pentobarbitone (synonym: pentobarbital). It was also found that pentobarbitone was effective at reducing motor signs in rats caused by deltamethrin, but not the motor signs caused by cismethrin (Forshaw, P.J. 1993; Forshaw, P.J. 2000). The authors speculated that the CS-syndrome produced by certain pyrethroids may be due to their antagonizing actions at VGClCs.

Table 1: Relationship of pyrethroids in binary mixtures on calcium influx and endogenous L-glutamate released from rat brain synaptosomes.

Binary Mixtures	Functional Assay Results[a]	
	Calcium Influx	L-glutamate Release
α-Cyano + α-Cyano		
Deltamethrin[b,c] + **λ-Cyhalothrin**[b]	Additive	Less-than-additive
Deltamethrin[b,c] + **γ-Cyhalothrin**[b]	Additive	Additive
Deltamethrin[b,c] + *Cypermethrin*[b,c]	Additive	More-than-additive
Deltamethrin[b,c] + **Esfenvalerate**[b]	Additive	Additive
Deltamethrin[b,c] + *Fenpropathrin*[c]	Less-than-additive	More-than-additive
Deltamethrin[b,c] + *β-Cyfluthrin*[b,c]	-	More-than-additive
α-Cyano + non-Cyano		
Deltamethrin[b,c] + *S-Bioallethrin*[c]	Less-than-additive	More-than-additive
Deltamethrin[b,c] + **Permethrin**[b]	Additive	Additive
Deltamethrin[b,c] + Tefluthrin	Less-than-additive	Additive
non-Cyano + non-Cyano		
Permethrin[b] + *S-Bioallethrin*[c]	Less-than-Additive	Additive

[a] Indicates the functional relationship between pyrethroid binary mixtures determined using the % additivity equation.
[b] Pyrethroids in **bold** act as VGCC agonists.
[c] Pyrethroids in *italics* act as VGClC antagonists as determined by Burr and Ray (Burr, S.A. 2004).

Burr *et al.* subsequently showed (Table 2) that bioallethrin (T-syndrome, type I), β-cyfluthrin (CS-syndrome, type II), cypermethrin (CS-syndrome, type II), deltamethrin (CS-syndrome, type II), and fenpropathrin (T-syndrome, type II), significantly decreased open channel probability. Bifenthrin (T-syndrome, type I), bioresmethrin (T-syndrome, type I), cispermethrin (T-syndrome, type I), cisresmethrin (syndrome not classified, type I), cyfluthrin isomers 2 and 4 (T-syndrome, type I), λ-cyhalothrin (CS-syndrome, type II), esfenvalerate (CS-syndrome, type II), and tefluthrin (T-syndrome, type I) did not significantly alter open channel probability. Since esfenvalerate and λ-cyhalothrin were both α-cyano and CS-syndrome pyrethroids and ineffective in decreasing open channel probability, while fenpropathrin and bioallethrin, both T-syndrome and type II and I pyrethroids, respectively, it was concluded that actions at the VGClC could not alone account for the differences between the two types of poisoning syndromes and that there were likely additional sites of action regulating the neurotoxicological response (Burr, S.A. 2004). Table 2 summarizes the effects of Type I and II pyrethroids on decreasing open channel probability of voltage-gated maxi chloride channels in excised inside-out membrane patches from N1E-115 mouse neuroblastoma cells. Pyrethroids were considered to decrease the open channel probability of chloride channels if the open channel probability significantly decreased post-dose as compared to pre-dose (Burr, S.A. 2004).

The GABA receptor-ionophore complex has likewise been implicated as a molecular target contributing to the neurotoxicity of pyrethroids. Casida *et al.* initially determined that certain pyrethroids also exhibit stereospecificity at the GABA chloride channel (Casida, J.E. 1985; Lawrence, L.J. 1983). In radioligand [^{35}S] t-

Table 2: Comparison of type I and II pyrethroids on their effect of open channel probability in voltage-gated maxi chloride channels.

Pyrethroid (10 µM)	Type of Pyrethroid	Syndrome Elicited	Decreased Open Channel Probability ($P < 0.05$)
Deltamethrin	II[a,b,c,d]	CS[e]	Yes
Cypermethrin	II[a,b,c]	CS[c]	Yes
Fenpropathrin	II[a,d]	T[f]	Yes
β-cyfluthrin	II[b,d]	CS[b]	Yes
Bioallethrin	I[a,b]	T[f,g]	Yes
Bifenthrin	I[b]	Not Classified[b]	No
Cisresmethrin	I[a]	Not Classified	No
Cyfluthrin isomer 2	II	CS[h]	No
Esfenvalerate	II[a,c,d]	CS[c,g]	No
Cyfluthrin isomer 4	II	CS[h]	No
Tefluthrin	I	T[h]	No
λ-cyhalothrin	II[b,d]	T[h]	No
Bioresmethrin	I[c]	T[c]	No
Cispermethrin	I[a,c]	T	No

Note. Pyrethroids are ordered according to the significance of their effect as determined by their *P* value (Burr, S.A. 2004).

[a] (Gammon, D.W. 1982)
[b] (Soderlund, D.M. 2002)
[c] (Verschoyle, R.D. 1980)
[d] (Wright, C.D.P. 1988)
[e] (Barnes, J.M. 1974)
[f] (Lawrence, L.J. 1982)
[g] (Verschoyle, R.D. 1972)
[h] (Symington, S.B. 2005)

butylbicyclophosphorothionate ($[^{35}S]$TBPS) binding studies using rat brain synaptic membranes, only type II pyrethroids inhibited binding in a stereospecific manner by binding to a site closely associated with that of TBPS and picrotoxin (both noncompetitive $GABA_A$ receptor antagonists) within the chloride channel ionophore. They determined that the toxic isomers of cypermethrin, deltamethrin, and fenpropathrin (type II - cyanophenoxybenzyl esters), each at 5 μM, inhibited $[^{35}S]$TBPS binding, whereas pyrethroids (up to 10 μM) that lacked the α-cyano substituent, such as *S*-bioallethrin, did not inhibit $[^{35}S]$TBPS binding. Since no nontoxic stereoisomers inhibited $[^{35}S]$TBPS binding, it was suggested that the binding site in the receptor was stereospecific to the chiral center found in toxic pyrethroids (Lawrence, L.J. 1983). It has been shown subsequently that GABA receptor blockade is not nearly as sensitive to pyrethroids as are VGSCs that are assayed in the same preparation. Nevertheless, the potential contribution of GABA receptors to the cumulative risk of pyrethroids cannot be discredited (Soderlund, D.M. 2002).

Hossain *et al.* recently revealed that certain pyrethroids had differential effects on glutamatergic (excitatory) and GABAergic (inhibitory) neurons in the hippocampus of rats (Hossain, M.M. 2008).

Allethrin (type I) exhibited a dual effect where low doses resulted in an increase in L-glutamate release but high doses of the compound decreased L-glutamate release. Type II pyrethroids, cyhalothrin and deltamethrin, produced opposite effects. Cyhalothrin inhibited L-glutamate release while deltamethrin increased L-glutamate release, but in a dose-dependent manner.

When GABAergic neurons were examined, extracellular levels of GABA increased in the presence of allethrin at its high dose and cyhalothrin at all doses, but decreased in the presence of deltamethrin. Interestingly, tetrodotoxin (TTX), a sodium channel blocker, prevented the effect of allethrin and cyhalothrin at all doses on L-glutamate and GABA release, but only partially blocked the effects of the highest dose of deltamethrin on L-glutamate release. These findings suggested that the effect of deltamethrin on L-glutamate release was not completely sodium-dependent and that additional mechanisms of action at other ion channels may be responsible. Local infusion of the L-type calcium channel blocker, nimodipine, completely blocked deltamethrin-evoked L-glutamate release at its highest concentration. These data collectively suggest that the excitatory glutamatergic neurons in the hippocampus are modulated by inhibitory GABA-releasing interneurons and confirm previous findings that multiple mechanisms are likely involved with the neurotoxic action of pyrethroids (Hossain, M.M. 2008).

In summary, certain binary mixtures of pyrethroids produce a more-than-additive effect on L-glutamate release at presynaptic nerve terminals that is not calcium-dependent. In a separate study, all pyrethroids in the four more-than-additive binary mixtures were also found to decrease the open channel probability of VGClCs, suggesting that these channels may play a role in the more-than-additive response previously observed in rat brain synaptosomes. In addition, pyrethroids exhibit stereospecificity at a binding site within the GABA chloride channel, the major regulator of inhibitory neurotransmitter release, further establishing the role of chloride channels in pyrethroid neurotoxicity. Thus, we hypothesize that an additional action at chloride

channels may be responsible for the more-than-additive effect on L-glutamate release previously seen with the equipotent binary mixtures of pyrethroids (deltamethrin with *S*-bioallethrin, β-cyfluthrin, cypermethrin, and fenpropathrin). To test this hypothesis, specific chloride channel blockers (antagonists) in the presence of deltamethrin were expected to reproduce the more-than-additive response.

Synaptosomes are an ideal *in vitro* model system to use to study the effects of pyrethroids since many of the physiological characteristics of the intact presynaptic nerve terminal are retained including: K^+ -stimulated Ca^{2+} influx and subsequent Ca^{2+} -dependent endogenous neurotransmitter release (L-glutamate) (Symington, S.B. 2007b). Additionally, synaptosomes are easily prepared, applicable to a variety of physiological measurements using fluorescent spectrophotometry, and are fully functional for a period of a few hours after preparation.

Since chloride channels generally function as rectifying channels to stabilize the membrane potential (Clark, J.M. 1995), it is expected that blocking these channels in the presynaptic nerve terminal, will increase neurotransmitter release. Furthermore, since deltamethrin targets VGSCs, VGCCs, and VGClCs (Burr, S.A. 2004), it is expected that deltamethrin at its EC_{50} value in the presence of a chloride channel antagonist will reproduce the more-than-additive effect. Lastly, concentrations of the four binary mixtures of pyrethroids that produced more-than-additive effects on L-glutamate release (Frisbie, R.K. 2006) should be shifted to lower concentrations in the presence of a chloride channel antagonist. In the presence of a chloride channel agonist, the more-than-additive response should be shifted to higher concentrations.

14

Antagonists and agonists were chosen based on their specificity to the many different types of chloride channels found in presynaptic nerve terminals. Anthracene-9-carboxylic acid (9-AC) and rChlorotoxin (ClTx) are VGClC and calcium-activated chloride channel (CaClC) antagonists. 4,4' - dinitrostilbene - 2,2' - disulfonic acid (DNDS) is a volume-regulated anion channel (VRAC) antagonist and 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB) is a VGClC, CaClC, and VRAC antagonist. Picrotoxinin is an antagonist of the GABA-gated chloride channel while pentobarbital and muscimol are GABA-gated chloride channel agonists.

Because there may be significant aspects of pyrethroid toxicity that were not considered in their original evaluations, findings from these experiments are expected to provide valuable insight into the assessment of the cumulative risk of pyrethroids and therefore their regulation under the FQPA.

1.2 Voltage-Gated Calcium Channels

Of the many channels and pumps that control intracellular calcium levels, voltage-gated calcium channels (VGCCs) play a key role in calcium homeostasis (Perez-Reyes, E. 2003). Depolarization-mediated calcium influx through VGCCs elicits a wide range of cytoplasmic responses that includes contraction of cardiac muscle, cellular proliferation, activation of calcium-dependent enzymes, and the release of hormones and neurotransmitter molecules (Wheeler, D.B. 1994; Tedford, H.W. 2006; Evans, R.M. 2006). Several subtypes of VGCCs have been identified based on their pharmacological and electrophysiological properties (termed L-, N- P-, Q-, R-, or T-types) with the predominant diversity lying within the pore-forming α_1 subunit (Evans, R.M. 2006).

Although each subtype functions to allow the passage of calcium ions into excitable cells, they differ in their cellular expression pattern, subcellular distribution, and the cytoplasmic messenger molecules that regulate them (e.g. protein kinases and G proteins) (Jarvis, S.E. 2001; Tedford, H.W. 2006). Calcium not only functions as a second messenger, but its entry into the cell can cause depolarization and activate other voltage-gated ion channels associated with the plasma membrane (Perez-Reyes, E. 2003). Calcium entry into these cells is therefore carefully regulated since excessive amounts of intracellular calcium are not only potentially toxic to the cell, but have also been linked to several disorders, and mutations and deletions in voltage-gated calcium channel genes have been associated with migraines, cerebellar ataxia, night blindness, and epilepsy (Jarvis, S.E. 2001; Lorenzon, N.M. 2000).

There are three families of VGCCs classified by the α_1 subunit gene subfamily (1-3). Calcium channels were named using the chemical symbol of calcium (Ca) with the principal physiological regulator of this channel (voltage) indicated as a subscript (Ca_V) (Catterall, W.A. 2003). Ca_v1 genes encode L-type channels, Ca_v2 genes encode N-type, P/Q type, and R-type channels, and Ca_v3 genes encode T-type channels (Evans, R.M. 2006). Table 3 summarizes the physiological function and pharmacology of VGCCs. For the purposes of this study, we focus on the Ca_v2 family of VGCCs, which are responsible for calcium entry into neurons and for calcium-mediated neurotransmitter release from presynaptic nerve terminals (Tedford, H.W. 2006).

The Ca_v2 subfamily ($Ca_v2.1$-3) includes channels containing α_{1A}, α_{1B}, and α_{1E} subunits, which allow P/Q-, N-, and R-type calcium currents, respectively. Alternative splicing of the $Ca_v2.1$ gene gives rise to the P- and Q-type channels (Catterall, W.A.

16

2000; Evans, R.M. 2006). Together the P/Q-type and N-type channels largely account for the rapid release of synaptic vesicles at the presynaptic nerve terminal following depolarization and calcium influx. The overall contribution of each, however, varies with the neuronal preparation (Burke, S.P. 1993; Potier, B. 1993; Tedford, H.W. 2006). The role of R-type channels is less well understood. They are localized to proximal dendrites and presynaptic nerve terminals, and thought to also contribute to the synaptic release of neurotransmitters (Tedford, H.W. 2006; Evans, R.M. 2006).

Table 3: Voltage-gated calcium channel subunit compositions and functions (adapted from Catterall 2003).

Ca^{2+} Channels	Ca^{2+} Current type	Primary Localizations	Cellular Functions
$Ca_V1.1$	L	Skeletal muscle	Excitation-contraction coupling Calcium homeostasis Gene regulation
$Ca_V1.2$	L	Cardiac muscle Endocrine cells Neurons	Excitation-contraction coupling Hormone secretion Gene regulation
$Ca_V1.3$	L	Endocrine cells Neurons	Hormone secretion Gene regulation
$Ca_V1.4$	L	Retina	Tonic neurotransmitter release
$Ca_V2.1$	P/Q	Nerve terminals Dendrites	Neurotransmitter release Dendritic Ca^{2+} transients
$Ca_V2.2$	N	Nerve terminals Dendrites	Neurotransmitter release Dendritic Ca^{2+} transients
$Ca_V2.3$	R	Cell bodies Dendrites Nerve terminals	Ca^{2+} -dependent action potentials Neurotransmitter release Repetitive firing
$Ca_V3.1$	T	Cardiac muscle Skeletal muscle Neurons	Repetitive firing
$Ca_V3.2$	T	Cardiac muscle Neurons	Repetitive firing
$Ca_V3.3$	T	Neurons	Repetitive firing

1.3 Chloride Channels

Since the cloning and functional expression of the first member of the mammalian voltage-dependent chloride channel (CLC) family of chloride channels in 1990 by Jentsch *et al*, chloride channels have become one of the most exciting topics in channel research. It is now well established that these channels function in several critical processes, including cell cycle and apoptosis, volume regulation, synaptic transmission, and cellular excitability (Nilius, B. 2003). Chloride channels are classified according to their mechanism of activation, and depends on: 1) transmembrane voltage (voltage-gated/dependent/sensitive channels, the ClC family), 2) cell swelling (volume-regulated anion channels, VRAC), 3) the binding of signaling molecules (ligand-gated anion channels of presynaptic and postsynaptic membranes, such as glycine- or γ-aminobutyric acid (GABA)- activated chloride channels, GAB_ACIC), 4) the phosphorylation of intracellular serine or threonine residues by protein kinases (cystic fibrosis transmembrane conductance regulator, CFTR), and 5) various ions (e.g., anions, H^+ (pH), or Ca^{2+} (Ca^{2+}- activated chloride channels, CaClC)) (Jentsch, T.J. 2002; Nilius, B. 2003). This classification scheme is not perfect, however, as some mechanisms of activation overlap (e.g. volume-regulated channels may also be voltage-dependent). It is likely that the final classification will be based on structural classes once more is learned about the structural similarities amongst channels (Jentsch, T.J. 1997).

There are currently three well-established gene families of chloride channels. In mammals, these include the largest known family, the ligand-gated GABA and glycine-receptor chloride channels, the CFTR family, and the CLC family (Jentsch, T.J. 1997). The plasma membrane of presynaptic nerve terminals possess ClCs, GABA/glycine-

gated chloride channels, and the less well understood CaClCs (Meir, A. 1999) and hence were the focus of this investigation.

The CLC has nine different genes in mammals that can be grouped into three clades based on sequence homology (Fig. 2) (Jentsch, T.J. 1993). The first clade comprises plasma membrane channels ClC-1, ClC-2, ClC-Ka, and ClC-Kb, which are close homologues of the prototype *Torpedo marmorata* channel ClC-0 and are involved in the stabilization of membrane potential and transepithelial transport. Members of the other two clades reside in intracellular membranes and have been implicated in the acidification of intracellular compartments such as endosomes and lysosomes. These include ClC-3, ClC-4, and ClC-5, and ClC-6 and ClC-7, respectively (Pusch, M. 2006; Jentsch, T.J. 2005).

Although most channel proteins in the CLC family show voltage-dependent gating, ClC-2 channels are highly expressed in specific regions of the brain and are activated by hyperpolarized potentials, cell swelling, and acidic pH, all important features of neuronal excitability (Thiemann, A. 1992; Grunder, S. 1992; Jordt, S.E. 1997).

	Expression	**Function**
ClC-0		
ClC-1	Skeletal muscle	Stabilization of plasma membrane V
ClC-2	Broad	Transepithelial transport? pH, volume regulation
ClC-Ka	Kidney, inner ear	Transepithelial transport
ClC-Kb	Kidney, inner ear	Transepithelial transport
ClC-3	Broad (brain, kidney, liver…)	Acidification of endosomes, synaptic vesicles
ClC-4	Broad (brain, muscle…)	?
ClC-5	Kidney (intestine, liver…)	Acidification of endosomes
ClC-6	Broad	?
ClC-7	Broad	Acidification of lysosomes, resorption lacuna of osteoclasts.

Figure 2: The ClC family of chloride channels in mammals. The dendogram denotes the similarity between the different genes. Distribution of genes in tissue and suggested functions are indicated. Adapted from (Jentsch, T.J. 1997).

The ligand-gated ion channel (LGIC) superfamily also known as ionotropic receptors, are regulated by the binding of chemical messengers, as opposed to the voltage- or stretch-activated channels. The LGIC superfamily is comprised of γ-aminobutyric acid (GABA), glycine (GlyR), nicotinic acetylcholine (nAChR), and the 5-hydroxytryptamine type 3 (5-HT$_3$) receptors (Betz, H. 1990; Macdonald, R.L. 1994; Kash, T.L. 2004).

In the central nervous system (CNS), GABA modulates inhibitory synaptic transmission by activating three classes of receptors: $GABA_A$, $GABA_B$, and $GABA_C$ receptors. Of these, $GABA_A$ and $GABA_C$ belong to the LGIC family. $GABA_B$ receptors belong to the G protein-coupled receptors (metabotropic receptors) (Meir, A. 1999). The binding of GABA to the extracellular domain (ECD) causes a rapid allosteric transition in the ion-conducting pore within the transmembrane domain (TMD) (Chakrapani, S. 2004). The resulting chloride influx hyperpolarizes the neuron and inhibits neuronal activity (Jentsch, T.J. 2002).

Chloride currents that are mediated by cytosolic calcium levels are termed calcium-dependent or calcium-activated chloride channels (also known as CaCC, CaClC or (Cl)Ca) (Hartzell, C. 2005). CaClCs have been identified in many cell types, including epithelial cells, neurons, cardiac and smooth muscle cells, and blood cells (Eggermont, J. 2004; Hartzell, C. 2005). Likewise, they have been implicated in a variety of cellular functions that include fertilization of oocytes, transepithelial fluid transport, and membrane excitability in cardiac muscle and neurons (Hartzell, C. 2005). Although their functions in neurons remain poorly established, it is thought that they are involved in membrane repolarization, the generation of after-potentials, and membrane oscillatory behavior (Hartzell, C. 2005). In most cells, the resting membrane potential is more negative than the chloride equilibrium potential, so when intracellular calcium, ($[Ca^{2+}]_i$), rises, chloride is effluxed from the cell causing a depolarization (Hartzell, C. 2005) and it is thought that CaClC-induced depolarization may also activate VGCCs (Nilius, B. 2003). Alternatively, if the chloride equilibrium potential is below the membrane

potential, then opening of CaClCs can lead to hyperpolarization as chloride fluxes in (Hartzell, C. 2005).

As their name implies, activation of CaClCs requires a rise in $[Ca^{2+}]_i$, which can come from either intracellular stores or influx of extracellular calcium (Hartzell, C. 2005). In some cases, calcium channels are coupled to CaClCs, such as in mouse sympathetic neurons, where L- and P-type VGCCs activate CaClCs (Martinez-Pinna, J. 2000). CaClC activation by calcium occurs via two general mechanisms: calcium can either bind to the channel protein directly or act indirectly on the channel via Ca^{2+}-binding proteins (e.g. calmodulin) or Ca^{2+}-dependent enzymes (e.g. calmodulin-dependent protein kinase of CamKII) (Hartzell, C. 2005).

1.4 Pharmacology of Chloride Channels

Ion channel modulators have important medical ramifications. Additionally, they are also widely used to isolate ion channel proteins, to distinguish between gating states, to investigate pore structure, and more commonly, to selectively suppress or enhance one type of ion channel out of a mixture of currents. Unfortunately, this is often difficult to do with anion channel modulators due to their inability to completely suppress ion flux. Because of their low potency, the high concentrations utilized generally result in undesirable side effects on ion transporters and components of intracellular signaling pathways. Lastly, many anion channel blockers lack selectivity making interpretations from pharmacological studies more complicated (Jentsch, T.J. 2002; Hartzell, C. 2005).

Table 4 provides the pharmacology profiles of the mammalian neuronal chloride channels examined in this study. As mentioned previously, many chloride channels overlap in their mechanisms of activation, and are likewise affected by a variety of nonspecific modulators.

Table 4. Pharmacology of mammalian chloride channels in nerve preparations.

Inhibitor Type	Inhibitor	ClC-2	CaCC	VRAC	GABA$_A$ ClC
Anthracene carboxylates	9-AC	√[a,b]	√[d]		
Arylaminobenzoates	NPPB	√[a,b,c]	√[e,f]	√[g]	
Disulfonic stilbene	DNDS			√[g]	
Protein toxin	ClTx	√[c]	√[f]		
Polycyclic epoxylactone	PTX				√[h]

[a] (Furukawa, T. 1998)
[b] (Clark, S. 1998)
[c] (Thompson, C.H. 2005)
[d] (De Castro, F. 1997)
[e] (Hartzell, C. 2005)
[f] (Dalton, S. 2003)
[g] (Abdullaev, I.F. 2006)
[h] (Zhang, S.J. 1993)

1.5 Role of Chloride Channels in Neurotransmitter Release

Chloride is the most abundant extracellular physiological anion in animal cells and plays a major role in stabilizing the membrane potential of excitable cells. In nerve terminals, this stabilizing function of chloride channels is involved with the inhibition of neurotransmitter release (Meir, A. 1999). Since the concentration of chloride in the cytoplasm is lower than outside of the cell, the chloride equilibrium potential (E_{Cl}) is

generally close to the resting membrane potential (Grunder, S. 1992; Smith, R.L. 1995; Meir, A. 1999).

Upon excitation of the membrane by depolarization due to sodium and/or calcium influx, chloride channels oppose neuronal excitability by rectifying or repolarizing the membrane potential by fluxing chloride ions into the cell, thus preventing the further release of neurotransmitters (Hille, B. 2001; Suzuki, M. 2006). VGClCs are largely responsible for rectifying the excitable membrane potential. ClC-2 in particular are activated by hyperpolarization, deactivated by depolarization, and show no time-dependent inactivation (Staley, K. 1994; Smith, R.L. 1995).

Fast inhibitory neurotransmission in the mammalian nervous system is mediated by the neurotransmitter GABA. In presynaptic nerve terminals, GABA-mediated chloride current is believed to inhibit neurotransmitter release via two mechanisms. The first is by retardation in the propagation of action potentials onto the nerve terminal, and second, by a decrease in the amount of neurotransmitter released by each action potential that reaches the terminal. This inhibition occurs by shunting membrane currents and reducing the probability of other ion channels (sodium and calcium) opening (Jackson, M.B. 1995; Meir, A. 1999). Another mechanism by which chloride channels regulate presynaptic inhibition has been proposed by a mathematical model that suggests depolarization, in association with an increase in outward chloride conductance, may inactivate other ion channels in the nerve terminal such as calcium and sodium channels (Graham, B. 1994).

CaClCs have also been implicated in the regulation of neurotransmitter release. Although CaClCs are expressed in a variety of different neurons, their functions remain

poorly established (Hartzell, C. 2005). Thus far, it is believed that CaClCs modulate excitability by generating afterpotentials, which in some neurons, may be sufficient to trigger spontaneous firing. It has been established that there are three factors that dictate the direction of chloride through CaClCs: the membrane potential, chloride concentration gradient, and the intracellular calcium concentration. When intracellular calcium increases either due to release from intracellular stores or influx via VGCCs, CaClCs are activated and cause chloride to exit the cell, resulting in a depolarization. This depolarization can increase the open channel probability of VGCCs resulting in additional calcium influx, thus further enhancing the depolarization, and ultimately neurotransmitter release. CaClCs can also lead to hyperpolarization when the membrane potential is more positive than the E_{Cl} (Hartzell, C. 2005). See section 1.7 for regulation of chloride channels.

1.6 Regulation of Chloride Channels

Given the ubiquitous expression and numerous physiological functions that chloride channels possess, it is no surprise that they are likewise regulated or activated by many mechanisms. As previously mentioned in section 1.3, chloride channels are classified according to their mechanism of activation. For the purposes of this study, the mechanisms of activation for ClC-2, GABA ClCs, and CaClCs found in presynaptic nerve terminals will be discussed.

ClC-2 belong to the family of voltage-gated chloride channels. Since many VGClCs function to rectify the membrane potential, the intracellular concentration of

chloride is always lower than the extracellular concentration and E_{Cl} is still above the resting membrane potential (RMP) (Hille, B. 2001). These channels would be expected, when activated by hyperpolarization, to be inwardly rectifying with significant conductance ($G_{Cl(v)}$) only at membrane potentials more negative than E_{Cl}, ultimately repolarizing the cell during an action potential and reducing excitability (Staley, K. 1994; Smith, R.L. 1995). In nerve terminals, the activation of VGClCs is associated with presynaptic inhibition since the decreased efficiency of subsequent inward currents to produce a depolarization reduces neurotransmitter release (Rudomin, P. 1990; Meir, A. 1999). It was determined that ClC-2 cDNA cloned from rat brain and heterologously expressed in *Xenopus laevis* oocytes displayed a similar voltage dependence to $G_{Cl(v)}$ and that both ClC-2 and $G_{Cl(v)}$ slowly activate at hyperpolarized potentials (-90 mV), deactivate at depolarized potentials (-30 mV), and show no time-dependent inactivation (Thiemann, A. 1992; Grunder, S. 1992; Staley, K. 1994).

Phosphorylation of ClC-2 channels suggests another possible mechanism by which these channels are regulated. One group showed that PKA was able to phosphorylate ClC-2 *in vivo* and *in vitro*, but when heterologously expressed in *Xenopus*, they found that the current was not affected (Park, K. 2001). To determine if PKA was directly phosphorylating ClC-2 or doing so by acting through another kinase, PKC and CaMKII were examined for their ability to phosphorylate ClC-2. Their findings revealed that PKC (*in vivo* and *in vitro)* and CaMKII (*in vitro*) failed to phosphorylate ClC-2. Though these results suggest that PKA activation of rat ClC-2 *in vivo* is independent of chloride channel activity, the possibility that PKC or CamKII activation might indirectly regulate ClC-2 activity cannot be ruled out (Park, K. 2001). A mechanism by which PKC

may indirectly modulate ClC-2 activity is described by Hartzell *et al*. where an increase in intracellular calcium due to IP_3 activates CaClCs, which produces a depolarization and ultimately feeds back to open additional ClC-2 (Hartzell, C. 2005). Indirect phosphorylation of ClC-2 by CaMKII has not been elucidated in rat neuron preparations, but has been in other species and preparations (Hartzell, C. 2005). In another study, researchers showed that rat ClC-2 lacked consensus sites for cAMP-dependent phosphorylation, but rabbit and human ClC-2 (at different positions) had them (Jordt, S.E. 1997). Together, these findings suggest that regulation of ClC-2 may be cell-type or species-specific.

ClC-2 channels may also function to prevent excessive osmotic swelling. When cells are exposed to hypotonic solutions or intracellular hypertonic solutions, they begin to swell and then recover their normal volume by releasing enough salt to prevent further water entry (Hille, B. 2001; Grunder, S. 1992; Nilius, B. 1996). It is said that during cell swelling, depolarization due to the activation of this channel may also trigger exocytosis in cells that possess L-type calcium channels (Grunder, S. 1992). However, these events are likely not relevant to pyrethroid neurotoxicity since membrane depolarization and increased neurotransmitter release from isolated presynaptic nerve terminals is observed when these channels are blocked. The regulation of these channels by volume will therefore not be further discussed.

It has been shown that the voltage-dependent current via ClC-2 is enhanced at normal resting potentials by low extracellular pH (Jordt, S.E. 1997; Park, K. 2001). Also, a deceleration in exocytosis was observed when chloride entry into chromaffin granules was inhibited by NPPB, thereby impairing the acidification of the granule (Camacho, M.

2006). Since neither of these events are consistent with previous findings that suggest inhibition of chloride channels results in an increase in L-glutamate release from isolated presynaptic nerve terminals, the regulation of ClC-2 by pH will not be discussed further for its role in pyrethroid neurotoxicity and neurotransmission.

The GABA receptor chloride ionophore ($GABA_A$ClC) is a ligand-gated ion channel superfamily, is responsible for fast inhibitory neurotransmission in the mammalian nervous system. Binding leads to the opening of the chloride channel within the complex and chloride influx, ultimately hyperpolarizing the neuron and thereby inhibiting neuronal activity (Meir, A. 1999; Jentsch, T.J. 2002). It is also thought that activation of ClC-2 may help the ligand-gated chloride channels to yield hyperpolarizing currents since ClC-2 activity determines intracellular Na^+ and Cl^- concentration (Dinudom, A. 1993; Suzuki, M. 2006).

$GABA_A$ receptors are also modulated by phosphorylation. Porter *et al.* found that the $GABA_A$ receptor contains a consensus phosphorylation site on an intracellular domain of the channel for cAMP-dependent protein kinase (PKA). Using whole-cell and excised, outside out patch clamp on cultured mouse spinal neurons, PKA significantly reduced GABA-evoked chloride currents by reducing the channel opening frequency and they speculate that a reduction in receptor function may lead to increased neuronal excitability (Porter, N.M. 1990). These findings are consistent with previous studies on rat brain synaptoneurosomes where cAMP (an activator of PKA) decreased $GABA_A$ receptor-mediated chloride flux (Heuschneider, G. 1989).

Browning *et al.* determined that Ca^{2+}/phosphatidylserine-dependent protein kinase (PKC) and PKA also phosphorylate the $GABA_A$ receptor purified from rat brain, but

Ca^{2+}/calmodulin kinase II (CaM kinase II) does not (Browning, M.D. 1990). Shortly after these findings, another group found that the PKC activator, phorbol myristoyl acetate (PMA), but not the inactive analog, phorbol 12-mono-myristate (PMM), inhibited GABA-gated chloride currents in *Xenopus* oocytes expressing mouse brain mRNA (Leidenheimer, N.J. 1992). They also found that PMA inhibited GABA-gated chloride currents from GABA$_A$ receptors in oocytes coexpressing human $\alpha_1\beta_1\gamma_{2L}$ subunit cDNAs. In mouse brain microsacs, activators of PKC as well as inhibitors of protein phosphatases, reduced muscimol-stimulated ^{36}Cl$^-$ uptake by brain membrane vesicles (Leidenheimer, N.J. 1992). In all, these findings provide evidence that the function of GABA$_A$ receptors *in situ* can be inhibited by PKC phosphorylation.

CaClC activation requires a rise in intracellular calcium concentration ($[Ca^{2+}]_i$) which can come from calcium influx or calcium release from intracellular stores. When ($[Ca^{2+}]_i$ is below ~1 μM, calcium-activated chloride current ($I_{Cl,Ca}$) is both voltage and time dependent, however, when ($[Ca^{2+}]_i$ rises above ~1 μM, the voltage and time dependence disappears as the activation rate increases. Deactivation of CaClCs follows a time course that is voltage dependent (Hartzell, C. 2005).

Calcium can directly activate CaClCs by binding to the channel or indirectly activate the channel by interacting with calcium-binding proteins or calcium-dependent enzymes (Hartzell, C. 2005). One study using *Xenopus* oocytes injected with rat brain mRNA, showed that L-glutamate receptors indirectly regulated calcium-dependent chloride channels via an inositol 1,4,5-triphosphate (IP$_3$)-Ca^{2+} pathway. When L-glutamate was applied to the bath, whole-cell currents were evoked and reversed direction near the chloride equilibrium potential. Likewise, injection of IP$_3$ or calcium

into the oocyte caused an increase in intracellular free calcium regardless of extracellular calcium and the subsequent current exhibited a reversal potential near the chloride equilibrium potential. These results suggested that glutamate receptors activate endogenous chloride channels utilizing IP$_3$ which mobilizes the calcium in the cell that activates CaClCs (Oosawa, Y. 1989).

Additional evidence for activation of CaClCs by extracellular calcium was demonstrated by Martinez-Pinna *et al.* when the use of selective VGCC inhibitors revealed that L- and P-type VGCCs appear to selectively couple to CaClCs while N-type VGCCs couple with calcium-activated potassium channels (Martinez-Pinna, J. 2000; Hartzell, C. 2005).

To our knowledge, direct binding of calcium to CaClCs has not yet been reported in neuronal preparations.

1.7 Role of Chloride Channels in Pyrethroid Neurotoxicity

VGSCs have been well established for decades as a major target site for all pyrethroids, however, due to the variablity of pyrethroid structures and/or the bimodal effect of pyrethroids on mammals (T- vs CS-syndrome), it was suggested that there was either heterogeneity within the sodium channel targets (Soderlund, D.M. 2002) or that pyrethoids, specifically the type II pyrethroids, had additional sites of action (Burr, S.A. 2004). Among the different sites of action proposed, chloride ion channels are believed to contribute significantly to pyrethroid neurotoxicity. The first interaction of pyrethroids on ligand-gated chloride channels was reported by Leeb-Lundberg and Olsen in 1980. These findings established a stereospecific inhibition by deltamethrin on the binding of

[3H]dihydropicrotoxinin to the convulsant site (chloride channel) of GABA receptors in rat brain synaptic membranes (Leeb-Lundberg, F. 1980). Later Lawrence and Casida conducted a study on 37 pyrethroids and documented the inhibition of [35S]TBPS binding in rat brain synaptic membranes by the toxic isomers containing the α-cyano moiety, but not by the non-toxic isomers of the α-cyano compounds or by any pyrethroids lacking the α-cyano moiety (Lawrence, L.J. 1983). Gammon *et al.* likewise proposed that ligand-gated chloride channels were targets in both mouse and cockroach preparations (Gammon, D.W. 1982).

Collectively, these findings led to the hypothesis that type II pyrethroids were convulsants and caused the CS-syndrome by blocking GABA binding at the GABA receptor-ionophore complex and indirectly augmented the release of excitatory neurotransmitters due to the reduced inhibitory input to the nervous system. Several studies of α-cyano pyrethroids inhibiting GABA-stimulated chloride uptake into brain vesicles further confirmed these pyrethroids as antagonists at the mamamalian brain GABA receptors (Bloomquist, J.R. 1986; Abalis, I.M. 1986). However, chloride uptake was typically incomplete at maximally effective pyrethroid concentrations. In addition, electrophysiolgoical assays that assessed the relative sensitivity of GABA receptors and VSSCs in dorsal root ganglion neurons found that GABA receptors were much less senstive to the actions of deltamethrin than were VSSCs. In all, these findings indicated that the ligand-gated chloride channel was not sufficiently sensitive to pyrethroids to have an influence at doses relevant to systemic poisoning.

Not long after these findings, VGClCs were proposed as targets for pyrethroids when it was found that deltamethrin decreased the open channel probability of these

channels, which was similar to the action of the chloride channel antagonist, 9-AC, in excised, inside-out patches of N1E-115 neuroblastoma cells (Forshaw, P.J. 1993). Additional studies further confirmed VGClCs as an improtant site of action for certain type II pyrethroids in that deltamethrin and cypermethrin (type II pyrethroids) decreased the open channel probability of VGClCs, while the type I pyrethroid cismethrin did not (Ray, D.E. 1997). Interestingly, the chloride channel agonist, pentobarbitone, was effective against the type II motor signs caused by deltamethrin, but not against the type I motor signs produced by cismethrin (Forshaw, P.J. 2000). Since deltamethrin affected VGClCs at concentrations that were toxicologically relevant, it was proposed that type II pyrethroids may contribute to the bimodal nature of the pyrethroid poisoning syndromes. These effects of pyrethroids on these channels, however, have not been confirmed in insect preparations (Bloomquist, J.R. 2003).

To date, CaClCs have not been implicated in the acute neurotoxic action of pyrethroids in either mammalian or insect preparations. However, the existence of pharmacological crosstalk between different chloride channel types complicates the pharmacological discrimination between these channels, and thus, they cannot be overlooked for their contribution to pyrethroid neurotoxicity (Soderlund, D.M. 2002)

.

CHAPTER 2

MATERIALS AND METHODS

2.1 Animals

Retired (ex-breeder) female Sprague-Dawley rats (400-600 g and $\geq$ 8 months) were purchased from Charles River Laboratories (Wilmington, MA) and maintained at the Central Animal Care Facility, Morrill Science Center 4 South, University of Massachusetts-Amherst (Amherst, MA). Animals were accommodated in standard plastic animal cages (45 cm x 24 cm x 20 cm) at two per cage *ad libitum*, on a 12:12 hr day and night light cycle. Animal procedures were conducted in accordance with the Institutional Animal Care and Use Committee (IACUC) guidelines (Protocol #: 26-09-12).

2.2 Chloride Channel Ligands

Chloride channel blockers (antagonists); anthracene-9-carboxylic acid (9-AC), 4,4'-dintitrostilbene-2,2'-disulfonic acid (DNDS), 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB), and picrotoxinin (PTX) were purchased from Sigma-Aldrich Inc. (St. Louis, MO) and rChlorotoxin (ClTx) was purchased from Alomone labs (Jerusalem, Israel). Stock solutions of 9-AC, DNDS, and NPPB were made in dimethyl sulfoxide (DMSO) with the final serial dilutions in 95 % ethanol. All PTX stock solutions were solubilized in 95% ethanol. ClTx was made in a conventional buffer of 20 mM HEPES

(pH 7.0). New stock solutions were made monthly and stored at -20 ^{o}C. See Appendix A for the chemical structures of the chloride channel ligands used in this study.

2.3 Pyrethroids

Technical grade pyrethroids (Table 5) used in this study were provided by the Pyrethroid Working Group (PWG); a conglomerate of the major manufacturers of synthetic pyrethroids in the United States and United Kingdom that is comprised of Aventis CropScience (Dublin, NJ), Bayer Corporation (Kansas City, MO), DuPont Agricultural Products (Newark, NJ), FMC Corporation (Philadelphia, PA), Syngenta (Cheshire, UK) and Valent USA Corporation (Montvale, CA).

Pyrethroid stock solutions were prepared in DMSO and serial diluted as required. The final serial dilution was made in 95 % ethanol. Binary mixtures of pyrethroids were prepared in equipotent ratios of their respective EC_{50}s the day of experiments unless otherwise indicated. See Appendix B for the chemical structures of the pyrethroids used in this study.

Table 5. The isomeric composition of the pyrethroids used in this study.

Compound Name (Manufacturer)	CAS RN	Isomeric Composition
Cypermethrin[a] (FMC) (*S*)-α-cyano-3-phenoxybenzyl (1*R*,3*R*)-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate	52315-07-8	98.7%- (*S*)-α-cyano-, (*1R*)-*cis*-
Deltamethrin[a] (Aventis) (*S*)-α-cyano-3-phenoxybenzyl (1*R*,3*R*)-3-(2,2-dibromovinyl)-2,2-dimethylcyclopropanecarboxylate	52820-00-5	98.0%- (*S*)-α-cyano-, (*1R*)-*cis*-
Fenpropathrin[a] (Valent) (*RS*)-α-cyano-3-phenoxybenzyl 2,2,3,3-tetramethylcyclopropanecarboxylate	64257-84-7	99.7%- (*RS*)-α-cyano-
Tefluthrin[a] (Syngenta) 2,3,5,6-tetrafluoro-4-methylbenzyl (*Z*)-(1*R*,3*R*)-3-(2-chloro-3,3,3-trifluoroprop-1-enyl)-2,2-dimethylcyclopropanecarboxylate	79538-32-2	95.0%- (*Z*)-(*1R*)-*cis*-

[a] Structures for pyrethroids used in this study are illustrated in Appendix B.

2.4 Chemicals

Percoll™ was purchased from GE Healthcare Bio-Sciences Corporation (Piscataway, NJ). Amplex® UltraRed reagent was purchased from Invitrogen Corporation (Carlsbad, California). All other chemicals and enzymes were purchased from Sigma-Aldrich Inc. (St. Louis, MO) at the highest purity available.

2.5 Synaptosome Preparation

Synaptosomes were prepared from whole rat brain sans brain stem as previously described (Dunkley *et al*., 1986) with the following modifications. Rats were sacrificed by decapitation after CO_2-induced unconsciousness and whole brains removed. Brain tissue was homogenized using a 15 ml Teflon-glass homogenizer with a motor-driven pestle (Model K43, Tri-R Instruments, Rockville Centre, NY) for 4-5 cycles at 400 rpm (~2-3 min) in ice-cold sucrose buffer (0.32 M sucrose, 1 mM EDTA, and 5 mM tris-base, pH = 7.4) and the homogenate centrifuged at 900 g for 10 min at 4 °C. The supernatant was transferred and divided into two new tubes and centrifuged at 15,000 g for 30 min at 4 °C. The resulting crude mitochondrial pellets (P2) were gently resuspended manually in saline A buffer (125 mM NaCl, 5 mM KCl, 1.2 mM NaH_2PO_4, 1.2 mM $MgCl_2$, 5 mM $NaHCO_3$, 20 mM HEPES, pH = 7.4) with 10 mM glucose using a 5 ml Teflon-glass homogenizer. The P2 suspensions were transferred to the top of a discontinuous Percoll™ gradient (4%, 10%, 15%, and 23% Percoll™ in ice-cold sucrose buffer, pH = 7.4) and centrifuged at 32,500 g for 10 min at 4 °C. Of the 5 bands formed between the sucrose-percoll layers in the gradient, bands 3 and 4 (containing the purified synaptosomes), were

collected by aspiration and placed into 2 new tubes filled with saline A buffer containing glucose. After mixing, the washed synaptosomes were collected by centrifuging at 15,000 g for 10 min at 4 °C. The supernatant was decanted and the purified synaptosomal pellets were resuspended manually using a 5 ml Teflon-glass homogenizer into a total of ~4 ml saline A with glucose.

2.6 Synaptosome Protein Determination

Synaptosomal protein concentration was determined spectrophotometrically using the bicinchoninic acid (BCA) assay (Smith *et al.*, 1985). Protein induced color changes were measured with a UVMAX kinetic microplate spectrophotometer at $\lambda = 560$ nm (Molecular Devices, Sunnyvale, CA). Protein concentrations were extrapolated from a standard curve using bovine serum albumin (BSA) as the standard protein. The standard curve was constructed by plotting absorbance (O.D.) as a function of the amount of BSA and synaptosomal protein values were estimated from the linear regression of the data generated from the standards.

2.7 Neurotransmitter Release Assay

Endogenous neurotransmitter release from synaptosomes was determined using an enzyme-linked assay with Amplex[®] UltraRed for the detection of L-glutamate as previously described (Nicholls, D.G. 1987; Symington, S.B. 2005). Purified synaptosomes were diluted in saline A buffer with glucose to a final protein concentration of 2 mg/ml and incubated with the appropriate pretreatments (antagonists) and treatments (pyrethroids). Solvent concentration during the addition of an antagonist

did not 0.2% DMSO/ethanol or 1% water when added to synaptosomes for pretreatment (5 min at 37 $^{\circ}$C). Pretreatments were followed by treatment with 0.2 µl of a 1000-fold concentrated stock solution of each pyrethroid binary mixture in DMSO or DMSO alone as a solvent control (99.9 % DMSO for a total DMSO concentration of 0.2%) for 20 min at 37 $^{\circ}$C prior to the start of each assay. Treated synaptosomes were added to 1.4 ml of warmed (37 $^{\circ}$C) saline B buffer with 10 mM glucose (2 mM $CaCl_2$, 125 mM NaCl, 5 mM KCl, 1.2 mM $MgCl_2$, 20 mM HEPES, pH = 7.4) containing 50 µM Amplex$^{®}$, 0.04 U/ml glutamate oxidase, and 0.125 U/ml horseradish peroxidase and aliquoted (150 µl/well) to a 96-well microtiter plate. The plate was mixed for 5 sec and basal fluorescence monitored (0-2 min) (Exλ = 530 nm and Emλ = 591 nm) at 37 °C using a Gemini-*XS* SPECTRAmax$^{®}$ fluorescent microplate reader (Molecular Devices, Sunnyvale, CA) equipped with SOFTmax$^{®}$ Pro software, Version 3.1.2 for Windows-PC (Molecular Devices). Synaptosomes were subsequently depolarized by adding 5 µl aliquots of KCl stock solutions (4, 8, 20, 40, or 60 mM final assay KCl concentration) and the net increase in the amount of L-glutamate released was determined from fluorescence recordings at 30 min post-depolarization. The 30 min incubation ensures quantitative detection of L-glutamate released (Invitrogen Product Information A12221) that occurs during the first ~10 s of depolarization (Wennemuth, G. 2000).

The total L-glutamate content of synaptosomes was determined using the nonionic detergent, 0.5% Triton X-100. Percent L-glutamate release was then calculated as indicated by Equation 1 (Symington, S.B. 2007b).

Equation 1

$$\text{Percent Glutamate Released} = \left(\frac{\text{Glutamate released after depolarization - L-Glutamate released before depolarization}}{\text{Total glutamate content of synaptosomes}} \right) \times 100$$

Values for endogenous neurotransmitter (L-glutamate) released were extrapolated from a standard curve. The L-glutamate standard curve was set up exactly as the neurotransmitter release assay, except in the absence of synaptosomes. Serial dilutions of L-glutamate (0-450 pmoles) were added (5 μl/well) in place of synaptosomes to a 96-well microtiter plate and fluorescence monitored as above. A standard curve was constructed by plotting the relative fluorescence units (RFU) generated as a function of the amount of L-glutamate present.

2.8 Assessment of the Effects of Antagonists and Pyrethroids

The data were first fit to a linear regression (Equation 2) using GraphPad Prism Software (Version 3.02, GraphPad Inc., San Diego, CA) to determine if the relationship between increasing concentration of the antagonist and the effect on L-glutamate release had a slope that was significantly different from zero. Slopes that were not significantly different from zero as determined by Equation 2 were not further evaluated for a concentration-dependent response. If the slope was significantly different from zero, the data were fit to the polynomial first order (Equation 2) and the sigmoidal dose-response (variable slope) equation (Equation 3) using GraphPad Prism Software (Version 3.02,

GraphPad Inc., San Diego, CA), and compared with an F-test to determine which equation fit the data best. Data that best fit Equation 2 were considered non-sigmoidal and EC_{50} values not determined. Data that best fit Equation 3 displayed a concentration-dependent response and EC_{50} values were determined.

Pyrethroid EC_{50} values were determined from concentration-dependent response curves previously generated by Frisbie (Frisbie, R.K. 2006). These data were used to establish a potency ratio that equalizes the potency of each antagonist and pyrethroid used in the generation of each binary mixture.

Equation 2

$$y = mx + b$$

Where:

y = Independent variable of the function x.
m = Slope of the line.
b = Y-intercept of the line.
x = Independent variable of the function y.

Equation 3

$$Response = \beta_0 + (\beta_{max} - \beta_0) / (1 + 10^{(\log EC_{50} - X)(Hill\ Slope)})$$

*Where***:**

β_0= 0 (Constant).
β_{max} = Efficacy Value.
EC_{50} = Potency Value, effective concentration producing 50% maximal response.
Hill Slope = Slope of the concentration-response curves and describes binding characteristics. If the Hill Slope is far from 1, receptor-ligand binding does not follow the law of mass action for a single site of action, but suggests multiple binding sites. A positive (+) Hill Slope suggests cooperative receptor-ligand interaction and a negative (-) Hill slope suggests noncooperative receptor-ligand interaction.
X = Log concentration of pyrethroid.

41

Antagonist and pyrethroid interactions on L-glutamate release were assessed by measuring the percent additivity (% additivity), which provides an efficacy measurement of interactions in binary mixtures (Equation 4) as described (Martin *et al.*, 2003) with the following modifications (Frisbie, R.K. 2006).

Actual response values obtained for each binary mixture were compared to the theoretical response values to determine an additive, more-than-additive, or less-than-additive relationship between pyrethroids or toxin and pyrethroid binary mixtures (Frisbie, R.K. 2006).

Equation 4

$$\% \ \underline{\textbf{More}}^{a} \ \textbf{or} \ \underline{\textbf{Less}}^{b} \ \textbf{Additivity}^{c} = \left(\frac{\textbf{Actual Response - Theoretical Response}}{\textbf{Theoretical Response}} \right) \ \textbf{x} \ \ \textbf{100}$$

Where:

Theoretical Response = Assay response value of a toxin at its EC_{50} added to the assay response value of a pyrethroid at its EC_{50} or pyrethroid 1 at its EC_{50} added to the assay response value of pyrethroid 2 at its EC_{50}.

Actual Response = Assay response value of the binary mixture at each component's EC_{50}.

[a] A positive percent additivity value indicates a <u>more</u> than additive response.
[b] A negative percent additivity value indicates a <u>less</u> than additive response.
[c] A value of 0 indicates additive response.

2.9 Statistical Analysis of Data

Unless otherwise stated, mean values (± S.E.) for L-glutamate release were determined from at least three synaptosome preparations using the average values obtained from eight replicates per synaptosome preparation ($n \geq 3$). Statistical significance of L-glutamate released due to treatment was assessed by the unpaired t-test (GraphPad Prism Version 3.02) using $P < 0.05$ as a criterion for evaluation. For antagonist and binary mixture evaluations, statistically significant differences in L-glutamate release were assessed using unpaired t-tests (GraphPad Prism Version 3.02) at $P < 0.05$. A one-way ANOVA using Tukey's multiple comparison post-test was used to compare all possible combinations of treatments and determine statistical significance of L-glutamate released (GraphPad Prism Version 3.02) using $P < 0.05$ as a criterion for evaluation.

CHAPTER 3

EFFECTS OF SELECTED CHLORIDE CHANNEL ANTAGONISTS ON PYRETHROID-ENHANCED NEUROTRANSMITTER RELEASE FROM SYNAPTOSOMES

3.1 Effect of Chloride Channel Antagonists on L-Glutamate Release

Rat brain synaptosomes, depolarized with 20 mM KCl, were used to evaluate the effects of selected chloride channel antagonists on L-glutamate release in equipotent binary mixtures with either cypermethrin, deltamethrin, fenpropathrin or tefluthrin to determine if chloride channels are involved in the more-than-additive response previously reported by Frisbie (Frisbie, R.K. 2006). The EC_{50} values of the chloride channel antagonists were determined from concentration-dependent response curves (Fig. 3) whereas pyrethroid EC_{50} values were previously determined (Frisbie, R.K. 2006).

Three criteria were used to determine if the selected chloride channel antagonists elicited a concentration-dependent response on L-glutamate release. The data were initially fit to Equation 2 (Section 2.8) to determine a treatment effect (data generated a liner regression response that had a slope significantly different zero). If the data met this criterion, Equation 3 (Section 2.8) was used by fitting the data to the Hill equation to determine if there was a concentration-dependent response between L-glutamate release and the dose of the antagonist. Lastly, an *F*-test was used to determine the best fit of the data to either Equations 2 or 3.

Of the five antagonists tested, only NPPB elicited a concentration-dependent response curve that satisfied all three criteria (F-test, $F = 208.2$, $P < 0.0001$, respectively) (Fig. 3). The concentration-dependent response curve of 9-AC did not reach saturating conditions at its limit of solubility (~4 mM) (Weinreich, F. 2001), however, the slope was significantly different from zero as determined by Equation 2 and the data generated a concentration-dependent response as determined by Equation 3 (Fig. 3). Since GraphPad Prism (Version 3.02) was able to fit the data to Equation 3 and generate an EC_{50} value that was consistent with the literature (Weinreich, F. 2001) despite failure of the F-test (F-test, $F = 1.21$, $P = 0.5406$), 9-AC was used to in the presence of deltamethrin to determine if a more-than-additive response could be elicited.

ClTx and DNDS also increased L-glutamate release with slopes significantly different from zero (Equation 2). GraphPad Prism was able to fit the data to concentration-dependent response curves and extrapolate an EC_{50} value for both antagonists. These values were consistent with those reported by the vendor (Alomone Labs product information sheet) and in the literature (Dalton, S. 2003), respectively. Nevertheless, both antagonists were determined to fit the linear regression equation the best (F-test, $F = 0.339$ and 4.486, respectively and $P = 0.7468$ and 0.3167, respectively) (Fig. 3). The PTX data did not establish a concentration-response relationship that was significantly different from zero (Equation 2), and hence was not further examined in the remaining experiments.

Table 6 summarizes the effects of increasing concentrations of chloride channel antagonists on L-glutamate release. As judged by their estimated EC_{50}s, antagonists varied by ~ 3 orders of magnitude (7500-fold) from the least potent (9-AC, 2.4×10^{-4} M)

to the most potent (ClTx, 3.21 x 10^{-8} M). Data are reported as means ± SE of 3 replicates from 3 synaptosomal preparations for 9-AC, 7 replicates from 7 synaptosomal preparations for ClTx, 9 replicates from 6 synaptosomal preparations for DNDS, and 7 replicates from 4 synaptosomal preparations for NPPB.

In terms of maximal response (efficacy), however, NPPB was the most efficacious at eliciting L-glutamate release from synaptosomes followed by (in order of decreasing efficacy) ClTx, DNDS, and 9-AC (Table 6). Antagonists varied by 7.7- fold from the least efficacious (9-AC, 3.64 % ± 4.79) to the most efficacious (NPPB, 28 % ± 1.38).

Figure 3. Effect of increasing concentrations of chloride channel antagonists on L-glutamate release from K^+-depolarized synaptosomes. Anthracene-9-carboxylic acid (9-AC), rChlorotoxin (ClTx), 4,4'-dintitrostilbene-2,2'-disulfonic acid (DNDS), 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB), Picrotoxinin (PTX). KCl (20 mM) was used to depolarize synaptosomes. Concentration-dependent response curves were fit with the sigmoidal dose-response (variable slope) function in GraphPad Prism version 3.02. Data are reported as means ± SE of at least 3 replicates from multiple synaptosome preparations (n > 3) and presented as percents over solvent controls.
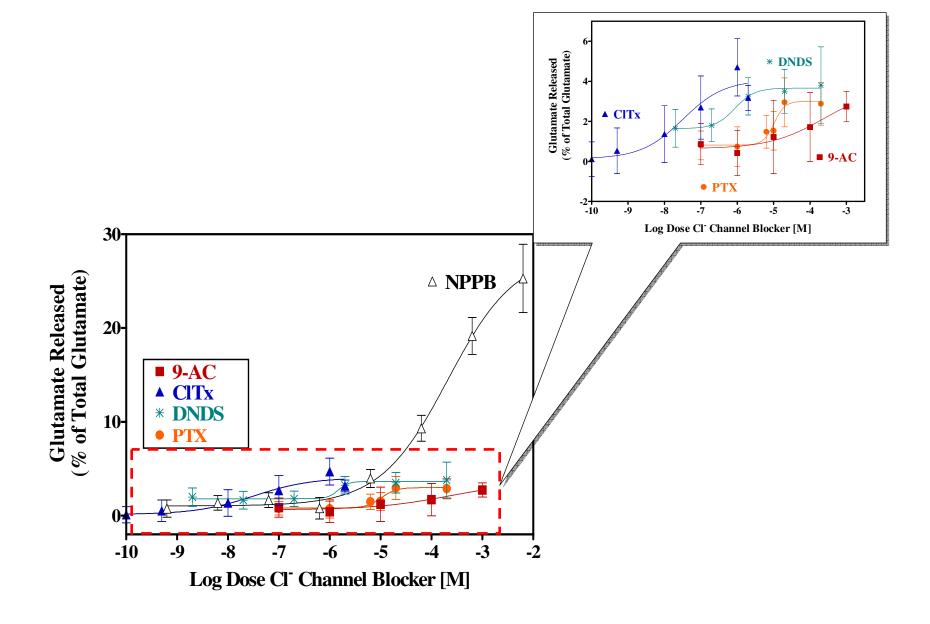
Table 6. The effects of increasing concentrations of chloride channel antagonists on L-glutamate release from rat brain synaptosomes.

	Hill Slope[a] (± SE)	EC$_{50}$ [M][a] (± 95% CI)	Maximum Response[a] (% Over Control) (± SE)	R^{2}[b]
Antagonists				
9-AC[c]	0.58 (±1.09)	2.4×10^{-4} (1.6×10^{-37} to $3.6 \times 10^{+29}$)	3.64 (± 4.79)	0.98
ClTx	0.76 (±0.88)	3.2×10^{-8} (5.1×10^{-11} to 2.0×10^{-5})	4.07 (± 1.15)	0.96
DNDS	1.60 (±0.81)	8.7×10^{-7} (1.1×10^{-9} to 7.0×10^{-6})	3.66 (± 0.15)	0.99
NPPB	0.65 (± 0.07)	2.3×10^{-4} (1.2×10^{-4} to 4.4×10^{-4})	28.0 (± 1.38)	0.99

[a] Estimates were derived from the Hill equation using GraphPad Prism (version 3.02).
[b] Correlation Coefficient.
[c] Saturation was not achieved at highest concentration examined.
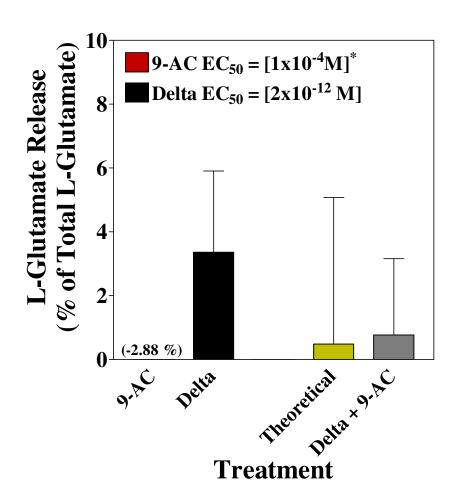
3.2 Effect of Pyrethroids in Equipotent Binary Mixtures with Chloride Channel Antagonists on L-glutamate Release from Synaptosomes

Chloride channel antagonists (9-AC, ClTx, DNDS, and NPPB) at their respective EC_{50} values were incubated with cypermethrin (type II, CS-syndrome), deltamethrin (type II, CS-syndrome), fenpropathrin (type II, T-syndrome), and tefluthrin (type I, T-syndrome) at their respective EC_{50} values to determine if chloride channels play a role in the more-than-additive effect previously seen with the four binary mixtures of pyrethroids reported by Frisbie (Frisbie, R.K. 2006).

9-AC (1×10^{-4} M) in the presence of deltamethrin (2×10^{-12} M) had no effect on L-glutamate release (Fig. 4). Compared to the theoretical mixture response (assay response value of synaptosomes in the presence of 9-AC alone at its EC_{50} added to the assay response value of deltamethrin alone at its EC_{50}), 9-AC in the presence of deltamethrin increased L-glutamate release 1.6-fold, however, this increase was not statistically significant and therefore was not considered to contribute to the more-than-additive response (unpaired t-test, $P = 0.9562$) (Fig. 4 and Table 7). Because none of the treatments were significantly different from each other (One-way ANOVA, Tukey's multiple comparison post-test, GraphPad Prism, Version 3.02) and since 9-AC did not elicit a more-than-additive response on L-glutamate release in the presence of deltamethrin, the remaining pyrethroids were not similarly tested.

Figure 4. Percent additivity of an equipotent binary mixture of 9-AC and deltamethrin (both at their respective EC_{50}s), on L-glutamate release from rat brain synaptosomes. Data are reported as means ± SE of 7 replicates from multiple synaptosome preparations (n = 3) and are presented as percents over the solvent control. An asterisk (*) indicates that concentration used in the assay was obtained from literature. Values in parentheses indicate a negative response on L-glutamate release.

Abbreviations: Delta = deltamethrin

L-Glutamate Release (% of Total L-Glutamate) vs Treatment

9-AC EC$_{50}$ = [1x10^{-4}M]*
Delta EC$_{50}$ = [2x10^{-12} M]

(-2.88 %)

9-AC   Delta   Theoretical   Delta + 9-AC

Treatment
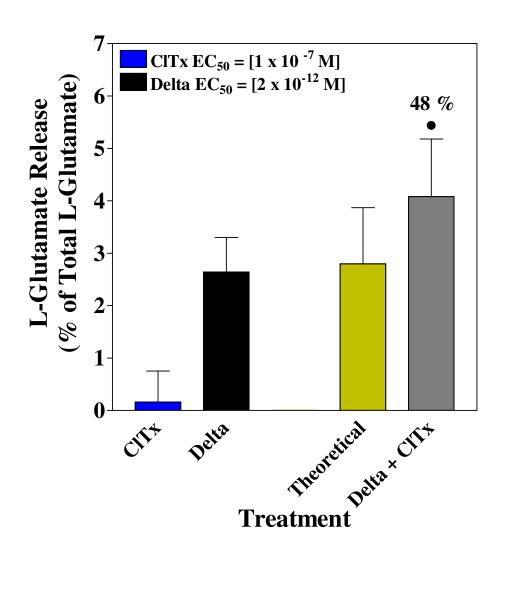
ClTx (1×10^{-7} M) in the presence of deltamethrin (2×10^{-12} M) significantly increased L-glutamate release 26-fold compared to synaptosomes treated with ClTx alone (unpaired t-test, $P = 0.004$). This release was increased 1.5-fold compared to synaptosomes treated with deltamethrin alone, but this increase was not significant (unpaired t-test, $P = 0.2727$) (Fig. 5). The actual response value of ClTx in binary mixture with deltamethrin increased L-glutamate release 1.5-fold compared to the theoretical mixture response, but the increase was not significant (unpaired t-test, $P = 0.4124$). Using the percent additivity equation (Equation 4), the actual response increased 48% over the theoretical. This increase, nevertheless, was not statistically significant indicating that a more-than-additive response was not generated (unpaired t-test, $P = 0.412$) (Fig. 5 and Table 7). Using the one-way ANOVA and Tukey's multiple comparison post-test, it was determined that none of the treatments were actually significantly different from each other, with the exception of ClTx plus deltamethrin compared to ClTx alone (GraphPad Prism, Version 3.02). Since ClTx did not elicit a more-than-additive response on L-glutamate release when in the presence of deltamethrin, the remaining pyrethroids were not similarly tested.

Figure 5. Percent additivity of an equipotent binary mixture of ClTx and deltamethrin, (both at their respective EC_{50}s), on L-glutamate release from rat brain synaptosomes. Data are reported as means ± SE of 9 replicates from multiple synaptosome preparations (n = 7) and are presented as percents over the solvent control. A closed circle (●) indicates that respective treatment is significantly greater than NT + ClTx treatment (unpaired t-test, $p < 0.05$, GraphPad Prism version 3.02).
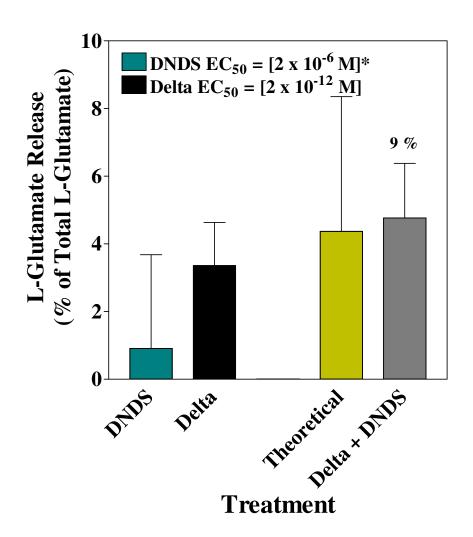
Abbreviations: Delta = deltamethrin

DNDS (2×10^{-6} M) in the presence of deltamethrin (2×10^{-12} M) increased L-glutamate release ~ 5-fold compared to synaptosomes treated with DNDS alone and 1.4-fold compared to synaptosomes treated with deltamethrin alone (Fig. 6). None of the increases were, however, significant (unpaired t-test, P = 0.2627 and 0.5111, respectively). The actual response value of DNDS in binary mixture with deltamethrin increased release 1.1-fold compared to the theoretical mixture response, however, this increase was not statistically significant. Thus a more-than-additive response was not generated (unpaired t-test, P = 0.9281) (Fig. 6 and Table 7). Furthermore, none of the treatments were significantly different from each other (One-way ANOVA, Tukey's multiple comparison post-test, GraphPad Prism, Version 3.02).

Figure 6. Percent additivity of an equipotent binary mixture of DNDS and deltamethrin (both at their respective $EC_{50}s$), on L-glutamate release from rat brain synaptosomes. Data are reported as means ± SE of 4 replicates from multiple synaptosome preparations (n = 4) and are presented as percents over the solvent control. An asterisk (*) indicates that concentration used in the assay was obtained from literature.

Abbreviations: Delta = deltamethrin

Legend (within figure):
DNDS EC_{50} = [2 x 10^{-6} M]*
Delta EC_{50} = [2 x 10^{-12} M]

y-axis: L-Glutamate Release (% of Total L-Glutamate)

x-axis: Treatment

Bar labels: DNDS, Delta, Theoretical, Delta + DNDS

9 %
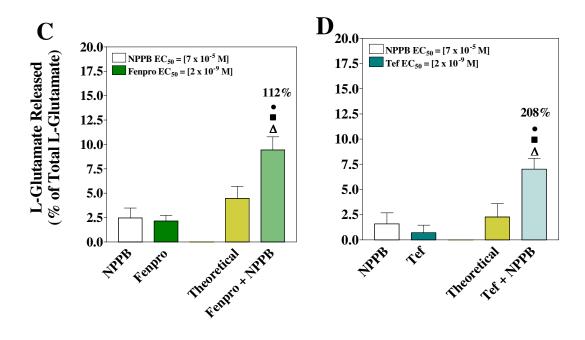
NPPB (7×10^{-5} M) in the presence of deltamethrin (2×10^{-12} M) (reported as means ± SE of 18 replicates from 7 synaptosomal preparations) significantly increased L-glutamate release 3.4-fold compared to synaptosomes treated with NPPB alone (unpaired t-test, $P < 0.0001$) and 3.5-fold compared t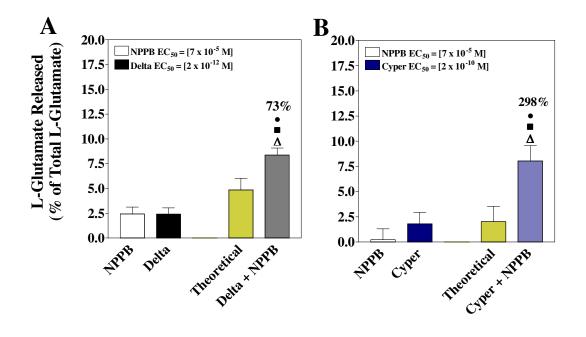o synaptosomes treated with deltamethrin alone (unpaired t-test, $P < 0.0001$) (Fig. 7A). The actual response value of NPPB in binary mixture with deltamethrin significantly increased L-glutamate release 1.7-fold compared to the theoretical mixture response (unpaired t-test, $P = 0245$). Using the percent additivity equation (Equation 4), the actual response increased 73% over the theoretical and was determined to have elicited a more-than-additive response on L-glutamate release (Fig 7A and Table 7). In addition, only the actual treatment of deltamethrin plus NPPB was determined to be significantly different from all other treatments (One-way ANOVA, Tukey's multiple comparison post-test, GraphPad Prism, Version 3.02).

To assess if this more-than-additive response was only generated by the NPPB plus deltamethrin mixture, NPPB was likewise examined in binary combinations with a slightly less potent type II pyrethroid that modifies VGSCs, VGCCS, and VGClCs, cypermethrin (Fig. 7B); a pyrethroid that is type II, but exhibits the T-syndrome and modifies VGSCs and VGClCs, fenpropathrin (Fig. 7C); and a type I pyrethroid that modifies VGSCs only, tefluthrin (Fig. 7D). Cypermethrin and NPPB (2×10^{-10} and 7×10^{-5} M, respectively) in binary mixture (reported as means ± SE of 4 replicates from 4 synaptosomal preparations) significantly increased L-glutamate release ~ 35-fold compared to synaptosomes treated with NPPB alone (unpaired t-test, $P = 0.0056$) and significantly increased L-glutamate release 4.5-fold compared to synaptosomes treated

Figure 7. Percent additivity of an equipotent binary mixture of NPPB and deltamethrin (A), cypermethrin (B), fenpropathrin (C), and tefluthrin (D), (both at their respective EC_{50}s) on L-glutamate release from rat brain synaptosomes. Data are reported as means ± SE of at least 3 replicates from multiple synaptosome preparations (n >3) and are presented as percent over the solvent control. A closed circle (●) indicates that respective treatment is significantly greater than NT + NPPB treatment (unpaired t-test, p < 0.05, GraphPad Prism version 3.02). An open triangle (Δ) indicates that respective treatment is significantly greater than pyrethroid treatment alone (unpaired t-test, p < 0.05, GraphPad Prism version 3.02). A closed square (■) indicates that respective treatment is significantly greater than theoretical mixture (unpaired t-test, p < 0.05, GraphPad Prism version 3.02).

Abbreviations: Delta = deltamethrin, Cyper = cypermethrin, Fenpro = fenpropathrin, and Tef = tefluthrin.

A

L-Glutamate Released
(% of Total L-Glutamate)

NPPB EC$_{50}$ = [7 x 10^{-5} M]
Delta EC$_{50}$ = [2 x 10^{-12} M]

73%

NPPB   Delta   Theoretical   Delta + NPPB

B

NPPB EC$_{50}$ = [7 x 10^{-5} M]
Cyper EC$_{50}$ = [2 x 10^{-10} M]

298%

NPPB   Cyper   Theoretical   Cyper + NPPB

C

L-Glutamate Released
(% of Total L-Glutamate)

NPPB EC$_{50}$ = [7 x 10^{-5} M]
Fenpro EC$_{50}$ = [2 x 10^{-9} M]

112%

NPPB   Fenpro   Theoretical   Fenpro + NPPB

D

NPPB EC$_{50}$ = [7 x 10^{-5} M]
Tef EC$_{50}$ = [2 x 10^{-9} M]

208%

NPPB   Tef   Theoretical   Tef + NPPB

Treatment

61

with cypermethrin alone (unpaired t-test, $P = 0.0166$) (Fig. 7B). The actual response value of NPPB in binary mixture with cypermethrin significantly increased 4-fold compared to the theoretical mixture response (unpaired t-test, $P = 0.0308$). Using the percent additivity equation (Equation 4), the actual response significantly increased L-glutamate release 298% over the theoretical mixture (Fig. 7B). In addition, only the actual treatment of cypermethrin plus NPPB was determined to be significantly different from all other treatments (One-way ANOVA, Tukey's multiple comparison post-test, GraphPad Prism, Version 3.02).

NPPB in the presence of fenpropathrin (7 x 10^{-5} and 2 x 10^{-9} M, respectively) (reported as means ± SE of 11 replicates from 6 synaptosomal preparations) significantly increased L-glutamate release 4-fold compared to synaptosomes treated with NPPB alone (unpaired t-test, $P = 0.0005$) and significantly increased L-glutamate release 4.4-fold compared to synaptosomes treated with fenpropathrin alone (unpaired t-test, $P < 0.0001$) (Fig. 7C). The actual response value of NPPB in binary mixture with fenpropathrin significantly increased 2-fold compared to the theoretical mixture response (unpaired t-test, $P = 0.0121$). Using the percent additivity equation (Equation 4), the actual response increased significantly 112% over the theoretical mixture (Fig. 7C). In addition, only the actual treatment of fenpropathrin plus NPPB was determined to be significantly different from all other treatments (One-way ANOVA, Tukey's multiple comparison post-test, GraphPad Prism, Version 3.02).

NPPB in the presence of tefluthrin (7 x 10^{-5} and 2 x 10^{-8} M, respectively) (reported as means ± SE of 7 replicates from 4 synaptosomal preparations) significantly increased L-glutamate release 4.4-fold compared to synaptosomes treated with NPPB

alone (unpaired t-test, $P = 0.0038$) and significantly increased L-glutamate release 10-fold compared to synaptosomes treated with tefluthrin alone (unpaired t-test, $P = 0.0004$) (Fig. 7D). The actual response value of NPPB in binary mixture with tefluthrin significantly increased 3-fold compared to the theoretical mixture response (unpaired t-test, $P = 0.0162$). Using the percent additivity equation (Equation 4), the actual response increased significantly 208% over the theoretical mixture (Fig. 7D). In addition, only the actual treatment of tefluthrin plus NPPB was determined to be significantly different from all other treatments (One-way ANOVA, Tukey's multiple comparison post-test, GraphPad Prism, Version 3.02).

Since tefluthrin modifies only VGSCs, the addition of a chloride channel antagonist such as NPPB should mimic the response seen with fenpropathrin, which modifies both VGSCs and VGClCs. When the assay response of fenpropathrin alone (2.15 % ± 0.57) was compared to the assay response of tefluthrin in an equipotent binary mixture with NPPB (7.03 % ± 1.07), it was found that the L-glutamate release increased by 3.3-fold and was statistically significant (unpaired t-test, $P = 0.0004$). When the assay response of tefluthrin in an equipotent binary mixture with NPPB was compared to the assay response of deltamethrin alone (2.41 % ± 0.64), L-glutamate release increased 2.9-fold and was statistically significant (unpaired t-test, $P = 0.003$). Likewise, when tefluthrin in the presence of NPPB was compared to cypermethrin alone (2.0 % ± 1.24), L-glutamate release significantly increased 3.5-fold (unpaired t-test, $P = 0.0261$).

Table 7 summarizes the effect of chloride channel antagonists at their estimated EC_{50}s on L-glutamate release in the presence of deltamethrin at its EC_{50} (2×10^{-12} M) on rat brain synaptosomes.

Table 7: Effect of chloride channel antagonists at their EC_{50}s on L-glutamate release from rat brain synaptosomes in the presence of deltamethrin at its EC_{50} (2×10^{-12} M).

	EC_{50}	Theoretical Response[a] ($\pm$ SE)	Actual Response[b] ($\pm$ SE)	Significantly Different ($p < 0.5$)	% Additivity[c]
Antagonist					
9-AC	0.1 mM	0.48% ($\pm$ 4.60)	0.77 % ($\pm$ 2.39)	No	-
ClTx	0.1 μM	2.80 % ($\pm$ 1.07)	4.08 % ($\pm$ 1.10)	No	48 %
DNDS	2 μM	4.37% ($\pm$ 3.98)	4.77% ($\pm$ 1.61)	No	-
NPPB	70 μM	4.85% ($\pm$ 1.17)	8.37% ($\pm$ 0.71)	Yes	73 %

[a] Theoretical response determined by the sum of the individual L-glutamate release response values in the presence of each pyrethroid alone at its EC_{50}.
[b] Actual response is the lowest concentration of pyrethroids in binary mixture observed that exhibited a more-than-additive response on L-glutamate release in the presence of NPPB (70 μM).
[c] Percent (%) additivity values for pyrethroid binary mixtures were calculated using Equation 4 (Section 2.8).

3.3 Discussion

Concentration-response curves were generated for four of the five chloride channel antagonists, 9-AC, ClTx, DNDS, and NPPB, all of which increased L-glutamate release from rat brain synaptosomes. The estimated EC_{50} values for 9-AC, ClTx, DNDS, and NPPB (2.39×10^{-4} M, 3.21×10^{-8} M, 1.65×10^{-6} M, and 2.30×10^{-4}, respectively) from our concentration response curves were consistent with previously established potency values in similar neuronal preparations (Weinreich, F. 2001; Clark, S. 1998; Dalton, S. 2003; Okada, Y. 2000).

PTX failed to elicit a treatment response on L-glutamate release that was significantly different from a slope of zero. Historically, the modulation of $GABA_ACICs$ by the inhibitory neurotransmitter, GABA, has been well established as occurring on postsynaptic targets (Jang, I.S. 2006). Recent evidence, however, suggests that these receptors are found also on presynaptic membranes (Zhang, S.J. 1993; Vautrin, J. 1994; Jang, I.S. 2006). Because of this, it is not clear why PTX was incapable of eliciting a concentration-dependent response on L-glutamate release from rat brain synaptosomes. Although the level of expression of these receptors is not known in our preparation, it is possible that the neurotransmitter release assay is too insensitive to detect the amount of L-glutamate release due to inhibition of $GABA_ACICs$. In addition, Vautrin *et al.* describe mechanisms by which synaptic transmission is mediated and seems likely that rat brain synaptosomes modulate transmission via presynaptic auto-transmission or "cis-mission" (Vautrin, J. 1994). If this were true, then it would be expected that both excitatory and inhibitory neurotransmitters would feedback on their respective receptors on the

presynaptic nerve terminal and an inability of one receptor to mediate synaptic transmission this way is expected to be the same for other receptors.

Using the established potency values for 9-AC, ClTx, DNDS, and NPPB, equipotent binary mixtures of the chloride channel antagonists and pyrethroids could be examined for a potential contribution of chloride channels to the more-than-additive response on L-glutamate release.

Deltamethrin was chosen as the initial pyrethroid to test because it has been established previously as the most potent pyrethroid on L-glutamate release from rat brain synaptosomes (Frisbie, R.K. 2006). Since the neurotoxic action of deltamethrin includes the modification of neuronal VGClC in addition to VGSC and VGCC (Chinn, K. 1986; Symington, S.B. 2005; Forshaw, P.J. 1990), it was hypothesized that blocking chloride channels with selective antagonists in the presence of deltamethrin should increase L-glutamate release and contribute to the more-than-additive response.

Collectively, our findings indicated that only NPPB was capable of significantly increasing L-glutamate release 73% when in equipotent binary mixtures with deltamethrin and reproduce the more-than-additive response previously seen with certain binary mixtures of pyrethroids (Frisbie, R.K. 2006). Although the equipotent binary mixture of ClTx and deltamethrin was not significantly different from the theoretical response, the percent additivity of this equipotent binary mixture was calculated (48%) for comparative purposes since ClTx was previously established as the most potent of all the tested antagonists. Additionally, the treatments were determined to be significantly different from each other using the one-way ANOVA and Tukey's multiple comparison post-test. 9-AC and DNDS did not significantly increase L-glutamate release in the

presence of deltamethrin and treatments were not determined to be significantly from each other. Hence, these compounds were not investigated further.

The effect of NPPB on L-glutamate release was investigated with three additional pyrethroids. Cypermethrin, like deltamethrin, has been shown to modify the gating kinetics of VGSCs, VGCCs, and VGClCs. Fenpropathrin modifies VGSCs and VGClCs, while tefluthrin modifies only VGSCs (Soderlund, D.M. 2002; Frisbie, R.K. 2006; Burr, S.A. 2004). In all three respective equipotent binary mixtures with cypermethrin, fenpropathrin, and tefluthrin, NPPB significantly increased L-glutamate release by 298, 112, and 208 %, respectively, when compared to the theoretical assay response and all treatments were significantly different from each other, as determined by the one-way ANOVA.

A more-than-additive response of L-glutamate release from rat brain synaptosomes occurred in the presence of binary mixtures of pyrethroids where at least one of the two pyrethroids increased neurotransmitter release and the other pyrethroid had an action on VGClCs (Frisbie, R.K. 2006). This phenomenon is likewise seen only in the combined presence of a pyrethroid that increases L-glutamate release and a chloride channel antagonist which blocks VGClCs in presynaptic nerve terminals, such as ClTx and NPPB, indicating that blockage of VGClCs may be necessary for the generation of the more-than-additive response.

CHAPTER 4

EFFECTS OF CHLORIDE CHANNEL ANTAGONIST ON NEUROTRANSMITTER RELEASE FROM SYNAPTOSOMES IN THE COMBINED PRESENCE OF EQUIPOTENT PYRETHROID BINARY MIXTURES

4.1 Effect of NPPB on Pyrethroid Binary Mixtures that Produced a More-Than-Additive Response on L-Glutamate Release

The potency (EC_{50}) value for NPPB on L-glutamate release was determined from concentration-dependent response data as 70 μM (Section 3.1) and used in the presence of two equipotent binary mixtures of pyrethroids (deltamethrin plus cypermethrin and deltamethrin plus fenpropathrin) that produced more-than-additive responses (Frisbie, R.K. 2006).

Figure 8A depicts the effects of deltamethrin and cypermethrin, individually and in binary mixture, on endogenous L-glutamate release (Frisbie, R.K. 2006). Synaptosomes were treated with either the EC_{50} of deltamethrin alone (2×10^{-12} M, final concentration), cypermethrin alone (2×10^{-10} M, final concentration) or with binary mixtures of deltamethrin and cypermethrin. Binary mixtures ranged from a 1:1 to 1:100 ratio of 2×10^{-12} M deltamethrin with increasing concentrations of cypermethrin (2×10^{-12} M to 2×10^{-10} M) (final total pyrethroid concentrations were 4×10^{-12} M to 2.02×10^{-10} M). A combined theoretical mixture response of deltamethrin and cypermethrin ($4 \times 10^{-}$
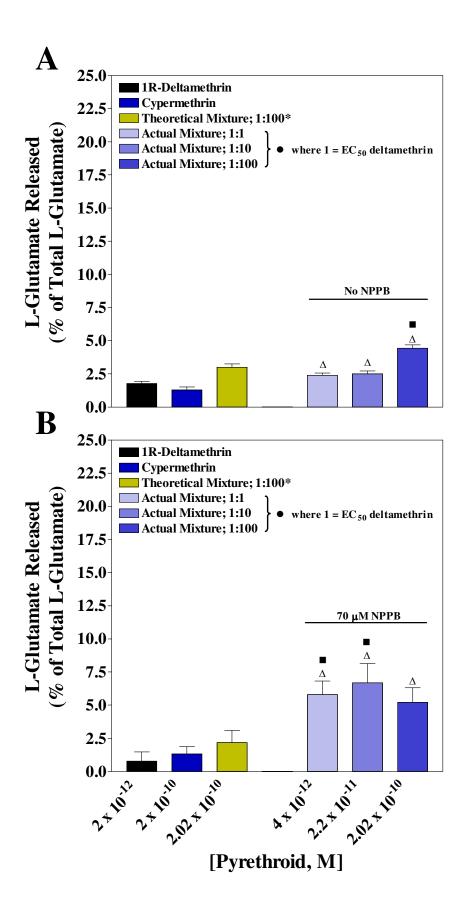
[12] M, final concentration) was generated by the addition of the assay response values for the two pyrethroids determined individually at their respective EC_{50} values.

Figure 8B depicts the effects of deltamethrin and cypermethrin individually and in binary mixture with 70 μM NPPB on endogenous L-glutamate release. Treatments were conducted as described above, but in the presence of 70 μM NPPB. All binary mixtures tested (1:1, 1:10, 1: 100), significantly increased (7.4-, 8.6-, 6.7-fold respectively) L-glutamate release compared to the deltamethrin treatment alone (0.78% ± 0.69) (unpaired *t*-test, *P* = 0.0036, 0.0062, and 0.0091, respectively).

When compared to the theoretical mixture, the deltamethrin plus cypermethrin actual mixtures (1:1, 1:10, 1:100) in the presence of NPPB increased L-glutamate release 2.7-, 3.1-, and 2.4-fold, respectively. However, only the 1:1 and 1:10 mixtures resulted in statistically greater release (unpaired *t*-test, *P* = 0.0293, 0.0300, and 0.0659, respectively).   Using the % additivity criteria (Equation 4), the 1:1 ratio of deltamethrin plus cypermethrin with NPPB at the 1:1 ratio (5.80 % ± 1.02) elicited a more-than-additive response that was 166 % more than the theoretical response (2.18 % ± 0.91) (Fig. 8B) . Thus, the more-than-additive response previously reported in the absence of NPPB (1:100) (Fig. 8A) (Frisbie, R.K. 2006) was shifted to a lower concentration of cypermethrin by one order of magnitude (1:10) (Fig. 8B)

Figure 9A depicts the effects of deltamethrin plus fenpropathrin, individually and in binary mixture, on endogenous L-glutamate release (Frisbie, R.K. 2006). Synaptosomes were treated with either the EC_{50} of deltamethrin alone (2 x 10^{-12} M, final concentration), fenpropathrin alone (2 x 10^{-8} M, final concentration) or with

Figure 8: Determination of additivity between deltamethrin and cypermethrin in the absence (A) (reproduced from Richard K. Frisbie M.S. thesis, University of Massachusetts, Amherst, © 2006) and presence (B) of NPPB (70 μM), on L-glutamate release from rat brain synaptosomes. Values reported are means ± SE of 10 replicates from multiple synaptosomal preparations (n = 5). KCl (20 mM) was used to depolarize synaptosomes. Synaptosomes were treated with either the EC_{50} of deltamethrin alone ([final assay] 2 x 10^{-12} M), the EC_{50} of cypermethrin alone ([final assay] 2 x 10^{-10} M) or a binary mixture in the presence of NPPB (70 μM). An asterisk (*) indicates the theoretical mixture response determined as the sum of the individual assay response values for deltamethrin and cypermethrin at their respective EC_{50} values. A closed circle (●) indicates the actual mixture response values for L-glutamate release in the presence of NPPB (70 μM) and binary mixtures composed of deltamethrin ([final assay] 2 x 10^{-12} M) and increasing concentrations of cypermethrin ([final assay] 2 x 10^{-12} to 2 x 10^{-10} M), ([final total pyrethroid assay] 4 x 10^{-12} to 2.02 x 10^{-10} M). An open triangle (Δ) indicates that the actual mixture is significantly greater than the deltamethrin treatment alone (unpaired t-test, $P < 0.05$). A closed square (■) indicates that actual mixture is significantly greater than the theoretical response (unpaired t-test, $P < 0.05$).
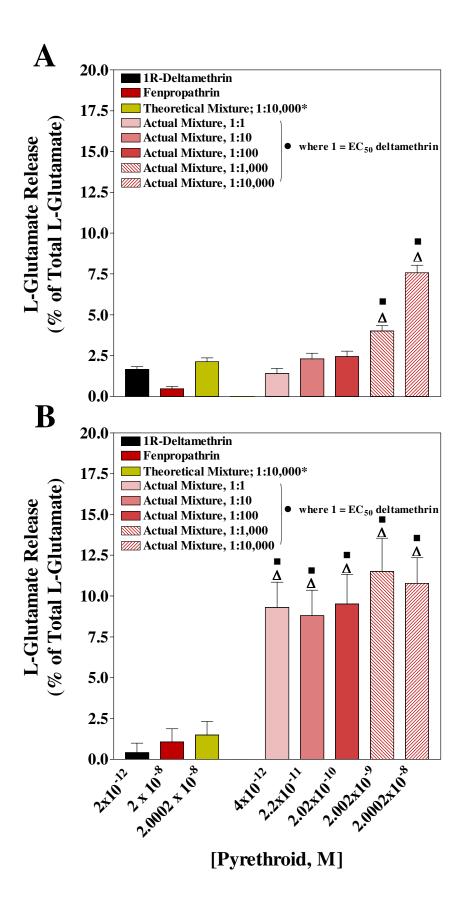
binary mixtures of deltamethrin and fenpropathrin in the presence of 70 μM NPPB. Binary mixtures ranged from a 1:1 to 1:10,000 ratio of deltamethrin to increasing concentrations of fenpropathrin (final total pyrethroid concentrations were 4×10^{-12} M to 2.0002×10^{-8} M). A combined theoretical mixture response of deltamethrin and fenpropathrin (4×10^{-12} M, final concentration) was generated as before.

Figure 9B depicts the effects of deltamethrin and fenpropathrin individually and in binary mixture with 70 μM NPPB on endogenous L-glutamate release. Treatments were conducted as described above, but in the presence of 70 μM NPPB. At all binary mixtures (1:1 to 1:10,000) tested, deltamethrin in the presence of increasing concentrations of fenpropathrin and 70 μM NPPB, significantly increased L-glutamate release (22-, 21-, 23-, 27-, and 26-fold, respectively) when compared to deltamethrin treatment alone (unpaired t-test, $P = 0.0007$, 0.001, 0.0014, 0.0008, and 0.0003, respectively).

Additionally, all binary mixtures (1:1 to 1:10,000) in the presence of NPPB significantly increased L-glutamate release (6.2-, 5.9-, 6.4-, 7.7-, 7.2-fold, respectively) when compared to the theoretical mixture (2.0002×10^{-8} M), (unpaired t-test, $P = 0.0021$, 0.0033, 0.0037, 0.0019, and 0.0008, respectively). Using the % additivity criteria (Equation 4), the 1:1 ratio of deltamethrin plus fenpropathrin with NPPB elicited a more-than-additive response that was 524 % more than the theoretical response (1.49 % ± 0.82).

Thus, the more-than-additive response previously reported in the absence of NPPB (1:1,000) (Fig. 9A) (Frisbie, R.K. 2006) was shifted to a lower concentration of fenpropathrin by three orders of magnitude (1:1) (Fig. 9B).

Figure 9: Determination of additivity between deltamethrin and fenpropathrin in the absence (A) (reproduced from Richard K. Frisbie M.S. thesis, University of Massachusetts, Amherst, © 2006) and presence (B) of NPPB (70 µM), on L-glutamate release from rat brain synaptosomes. Values reported are means ± SE of 5 replicates from multiple synaptosomal preparations (n = 3). KCl (20 mM) was used to depolarize synaptosomes. Synaptosomes were treated with either the EC_{50} of deltamethrin alone ([final assay] 2 x 10^{-12} M), the EC_{50} of fenpropathrin alone ([final assay] 2 x 10^{-8} M) or a binary mixture in the presence of NPPB (70 µM). An asterisk (*) indicates the theoretical mixture response determined as the sum of the individual assay response values for deltamethrin and fenpropathrin at their respective EC_{50} values. A closed circle (●) indicates the actual mixture response values for L-glutamate release in the presence of NPPB (70 µM) and binary mixtures composed of deltamethrin ([final assay] 2 x 10^{-12} M) and increasing concentrations of fenpropathrin ([final assay] 2 x 10^{-12} to 2 x 10^{-8} M), ([final total pyrethroid assay] 4 x 10^{-12} to 2.0002 x 10^{-8} M). An open triangle (Δ) indicates that the actual mixture is significantly greater than the deltamethrin treatment alone (unpaired t-test, $P < 0.05$). A closed square (■) indicates that the actual mixture is significantly greater than the theoretical response (unpaired t-test, $P < 0.05$).

4.2 Effect of NPPB in the Presence of a Pyrethroid Binary Mixture that Produced an Additive Response on L-Glutamate Release

To further validate a role of chloride channels in producing the more-than-additive response seen with certain binary mixtures of pyrethroids, the chloride channel antagonist, NPPB, was used in the presence of a binary mixture of pyrethroids that elicited an additive response on L-glutamate release. The potency (EC_{50}) value for the chloride channel antagonist, NPPB, was determined from a concentration-response curve (Section 3.1) and used in the presence of an additive binary mixture, deltamethrin plus tefluthrin (Frisbie, R.K. 2006).
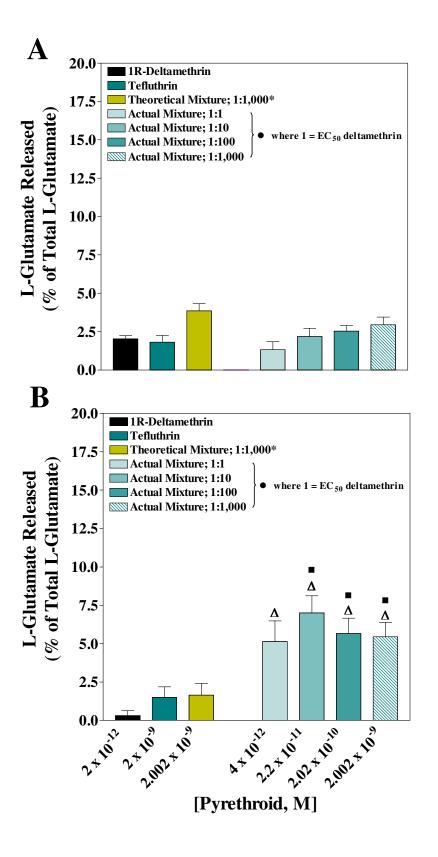
Figure 10A depicts the effects of deltamethrin and tefluthrin in binary mixture on endogenous L-glutamate release. Synaptosomes were treated with either the EC_{50} of deltamethrin alone (2×10^{-12} M, final concentration), the EC_{50} of tefluthrin alone (2×10^{-9} M, final concentration) or with binary mixtures of deltamethrin plus tefluthrin with 70 μM NPPB. Binary mixtures ranged from a 1:1 to 1:1,000 ratio of 2×10^{-12} M deltamethrin with increasing concentrations of tefluthrin (2×10^{-12} to 2×10^{-9} M final concentration) (final total pyrethroid concentrations were 4×10^{-12} M to 2.002×10^{-9} M). A combined theoretical mixture response of deltamethrin and tefluthrin (4×10^{-12} M, final concentration) was generated as before.

Figure 10B depicts the effects of deltamethrin and tefluthrin individually, and in binary mixture with 70 μM NPPB on endogenous L-glutamate release. Treatments were conducted as above, but in the presence of 70 μM NPPB. All binary mixtures (1:1 to 1:1,000) in the presence of NPPB significantly increased (17-, 23-, 19-, and 18-fold,

respectively) L-glutamate release when compared to the deltamethrin treatment alone (unpaired t-test, P = 0.0133, 0.0012, 0.0023, 0.0008, and 0.0022, respectively). When compared to the theoretical treatment, deltamethrin and tefluthrin in the presence of NPPB increased L-glutamate release (3.1-, 4.2-, 3.4-, and 3.3-fold, respectively), but only the mixtures from 1:10 to 1:1000 were significantly different from the theoretical response (unpaired t-test, P = 0.0075, 0.0188, and 0.0207, respectively). Using the % additivity criteria (Equation 4), deltamethrin and tefluthrin in the presence of NPPB at the 1:10 ratio (7 % ± 1.12) elicited a more-than-additive response that was 324 % more than 1:1,000 theoretical mixture (1.65 % ± 0.76).

Overall, these findings suggest that NPPB is capable of synergizing the effect of deltamethrin on L-glutamate release from rat brain synaptosomes. Previous studies have shown that deltamethrin and tefluthrin, when in binary combination, elicit only an additive response on L-glutamate release (Frisbie, R.K. 2006). However, the effect of these compounds when in binary mixtures, on L-glutamate release, can be enhanced by antagonizing the chloride channel.

Figure 10: Determination of additivity between deltamethrin and fenpropathrin in the absence (A) (reproduced from Richard K. Frisbie M.S. thesis, University of Massachusetts, Amherst, © 2006) and presence (B) of NPPB (70 μM), on L-glutamate release from rat brain synaptosomes. Values reported are means ± SE of 5 replicates from multiple synaptosomal preparations (n = 3). KCl (20 mM) was used to depolarize synaptosomes. Synaptosomes were treated with either the EC_{50} of deltamethrin alone ([final assay] 2 x 10^{-12} M), the EC_{50} of fenpropathrin alone ([final assay] 2 x 10^{-8} M) or a binary mixture in the presence of NPPB (70 μM). An asterisk (*) indicates the theoretical mixture response determined as the sum of the individual assay response values for deltamethrin and fenpropathrin at their respective EC_{50} values. A closed circle (●) indicates the actual mixture response values for L-glutamate release in the presence of NPPB (70 μM) and binary mixtures composed of deltamethrin ([final assay] 2 x 10^{-12} M) and increasing concentrations of fenpropathrin ([final assay] 2 x 10^{-12} to 2 x 10^{-8} M), ([final total pyrethroid assay] 4 x 10^{-12} to 2.0002 x 10^{-8} M). An open triangle (Δ) indicates that the actual mixture is significantly greater than the deltamethrin treatment alone (unpaired t-test, $P < 0.05$). A closed square (■) indicates that the actual mixture is significantly greater than the theoretical response (unpaired t-test, $P < 0.05$).

4.3 Discussion

In order to establish a role of chloride channel blockage in producing the more-than-additive response on L-glutamate release, NPPB, the most efficacious of the chloride channel antagonists identified in our study, was used in the presence of two more-than-additive pyrethroid binary mixtures and one additive pyrethroid binary mixture previously established by Frisbie.(Frisbie, R.K. 2006).

Since chloride channels generally function to rectify membrane potential (Hille, B. 2001), it was expected that blocking such channels in isolated presynaptic nerve terminals (synaptosomes) would increase membrane depolarization and cause an increase in L-glutamate alone.

In the presence of the binary mixtures that elicit a more-than-additive response (deltamethrin plus cypermethrin and deltamethrin plus fenpropathrin), it was expected that NPPB would shift the more-than-additive response to a lower combined concentration of the two pyrethroids in binary mixtures and that the more-than-additive response would increase in a concentration-dependent manner as seen by Frisbie. (Frisbie, R.K. 2006). In the presence of an additive binary mixture (deltamethrin plus tefluthrin), it was expected that a more-than-additive response on L-glutamate release would be generated, but that a concentration-dependent response would not be elicited.

Our findings indicate that chloride channels do play a role in eliciting a more-than-additive response.

Table 8 summarizes the relationship of binary mixtures of pyrethroids in the presence of 70 μM NPPB on endogenous L-glutamate release from rat brain

80

synaptosomes.   Deltamethrin in equipotent binary mixtures with cypermethrin, fenpropathrin, and tefluthrin, increased L-glutamate release (166, 524, and 324 % respectively) compared to the theoretical response.   These responses were all significantly different from the theoretical response, indicating a more-than-additive response has occurred as judged by the % additivity criteria (Equation 4).

Table 9 summarizes the results of adding NPPB to binary mixtures of pyrethroids that elicited either more-than-additive or additive responses and compared these effects to those elicited by binary mixtures without NPPB. Deltamethrin plus cypermethrin in the absence of NPPB first elicited a more-than-additive response at the 1:100 mixture. With NPPB, this response was first detected with the 1:1 mixture. Thus, the more-than-additive response was elicited at a 100-fold lower cypermethrin concentration and the amount of L-glutamate release was enhanced 2.4-fold compared to synaptosomes treated at the 1:100 mixture without NPPB.

Deltamethrin plus fenpropathrin in the absence of NPPB first elicited a more-than-additive response at the 1: 1,000 mixture. With NPPB, this response was first detected with the 1:1 mixture. Thus, the more-than-additive response was elicited at a 1000-fold lower fenpropathrin concentration and the amount of L-glutamate release was enhanced 6.6-fold compared to synaptosomes treated at the 1:1,000 mixture without NPPB.

Deltamethrin plus tefluthrin in the absence of NPPB did not elicit a more-than-additive response. With NPPB, a more-than-additive response was first detected with the

Table 8: Relationship of binary mixtures of pyrethroids in the presence of NPPB (70 μM) on endogenous L-glutamate release from rat brain synaptosomes.

| Pyrethroid Binary Mixture | Ratio[a] | L-glutamate Released (% of Total L-glutamate) | | % Additivity[d] |
		Theoretical Mixture Response[b] (± SE)	Actual Mixture Response[c] (± SE)	
Deltamethrin + Cypermethrin	1:1	2.18 (± 0.91)	5.8 (± 1.02)*	166 %
Deltamethrin + Fenpropathrin	1:1	1.49 (± 0.82)	9.30 (± 1.55)*	524 %
Deltamethrin + Tefluthrin	1:10	1.65 % (± 0.76)	7.0 % (± 1.12)*	324 %

[a] Observed potency ratio of pyrethroids in binary mixture that exhibited the more-than-additive response in the presence of NPPB (70 μM) where 1 equals the EC_{50} of deltamethrin (2×10^{-12} M).
[b] Theoretical response determined by the sum of the individual L-glutamate release response values in the presence of each pyrethroid alone at its EC_{50}.
[c] Actual response is the lowest concentration of pyrethroids in binary mixture observed that exhibited a more-than-additive response on L-glutamate release in the presence of NPPB (70 μM).
[d] Percent (%) additivity values for pyrethroid binary mixtures were calculated using Equation 4 (Section 2.8).
* An asterisk indicates that the actual mixture response is statistically different from the theoretical mixture response, unpaired t-test, $P < 0.05$ (GraphPad Prism).

Table 9: Comparison of binary mixtures in the absence and presence of NPPB.

Equipotent Binary Mixture	Assay Response [a,b] - NPPB (70 μM) (± SE)	Assay Response [a] + NPPB (70 μM) (± SE)	Fold-increase
Deltamethrin + Cypermethrin			
1:1	2.4 (± 0.17)	5.8 (± 1.02) *	2.4
1:10	2.5 (± 0.22)	6.7 (± 1.45)	2.7
1:100	4.4 (± 0.26)*	5.22 (± 1.10)	1.2
Deltamethrin + Fenpropathrin			
1:1	1.4 (± 0.31)	9.3 (± 1.55)*	6.6
1:10	2.3 (± 0.34)	8.8 (± 1.57)	3.8
1:100	2.4 (± 0.34)	9.5 (± 1.81)	4.0
1:1,000	4.0 (± 0.31)*	11.5 (± 2.04)	2.9
1:10,000	7.6 (± 0.46)	10.8 (± 1.58)	1.4
Deltamethrin + Tefluthrin			
1:1	1.3 (± 0.52)	5.1 (± 1.35)	3.8
1:10	2.2 (± 0.54)	7.0 (± 1.12)*	3.2
1:100	2.53 (± 0.37)	5.7 (± 1.00)	2.2
1:1,000	3.0 (± 0.50)	5.4 (± 0.95)	1.8

[a] Assay response is the amount of L-glutamate released in the presence of a binary mixture of pyrethroids with or without NPPB.
[b] Values obtained from work done by Frisbie (Frisbie, R.K. 2006).
* An asterisk indicates the lowest concentration at which a more-than-additive response L-glutamate release was observed.

1:10 mixture. The amount of L-glutamate release was enhanced 3.2-fold compared to synaptosomes treated at the 1:10 mixture without NPPB.

Overall, the more-than-additive binary mixtures (deltamethrin plus cypermethrin and deltamethrin plus fenpropathrin) in the presence of NPPB shifted this response to lower binary mixture concentrations and the additive binary mixture (deltamethrin plus tefluthrin) in the presence of NPPB elicited a more-than-additive response as expected. None of the pyrethroid binary mixtures in the presence of NPPB, however, elicited L-glutamate release in a concentration-dependent manner. It is possible that the concentration of NPPB used saturated certain chloride channels that are target sites for selected pyrethroids and hence a concentration effect on L-glutamate release could not be distinguished using the different ratios of pyrethroids in binary mixtures. Lower concentrations of NPPB should be tested to clarify this effect. In addition, since the additive binary mixture, deltamethrin plus tefluthrin with NPPB elicited a more-than-additive response on L-glutamate release, it is not clear whether this response is due to the action of NPPB at the chloride channels alone or if this inhibitor enhances the action of tefluthrin at VGSCs. Therefore, the VGSC inhibitor, tetrodotoxin (TTX), could be used in the presence of this pyrethroid binary mixture with NPPB to determine if the more-than-additive response is abolished. If this response is abolished, it suggests that modifying chloride channels ultimately modulates sodium conductance via VGSCs and this action is important also for pyrethroids that affect only VGSCs.

Similar to NPPB, ClTx in the presence of select binary mixtures can also enhance L-glutamate and shift the more-than-additive response to lower concentrations (Frisbie, R.K. 2006). Deltamethrin plus fenpropathrin in the absence of ClTx first elicited the

more-than-additive response at the 1:1,000 mixture. With ClTx, this response was first

detected with the 1:10 mixture (2.65 ± 0.26). Thus, the more-than-additive response was

elicited at a 100-fold lower fenpropathrin concentration and the amount of L-glutamate

release was enhanced 1.2-fold compared to synaptosomes treated at the 1:1,000 mixture

without ClTx. These findings were not surprising since NPPB and ClTx target the same

chloride channels (VGClC and CaClC).

CHAPTER 5

SUMMARY AND FUTURE DIRECTIONS

Thus far, our findings have implicated the importance of chloride channels in rat brain synaptosomes in the acute neurotoxicity response of pyrethroids. Since NPPB and ClTx are known inhibitors of ClC-2 VGClCs and CaClCs and have been shown to enhance L-glutamate release, we propose that these channels are present in our synaptosomal preparations and are modified by pyrethroids. PTX did not elicit a concentration-dependent response. It is not clear whether $GABA_A ClC$ are absent in our synaptosome preparation or if the level of expression is so low that our assay is not sensitive enough to detect any increase in L-glutamate release due to blockage of these channels.

There are at least two mechanisms by which pyrethroids are capable of eliciting increased L-glutamate release from presynaptic nerve terminals that involve blockage of the two chloride channels. In the first model, deltamethrin initially targets VGSCs and causes synaptolemma depolarization due to an influx of sodium ions (Fig. 11). VGCCs in active release zones activate in response to membrane depolarization and calcium influx is prolonged due to modification by deltamethrin (Symington, S.B. 2007b). At this time, the membrane potential has become significantly more positive than the E_{Cl}, and under normal circumstances, VGClCs activate allowing negative charge to flux into the synaptosome (hyperpolarization) and return the membrane potential back to RMP. In the presence of a pyrethroid that blocks VGClCs, however, the hyperpolarizing
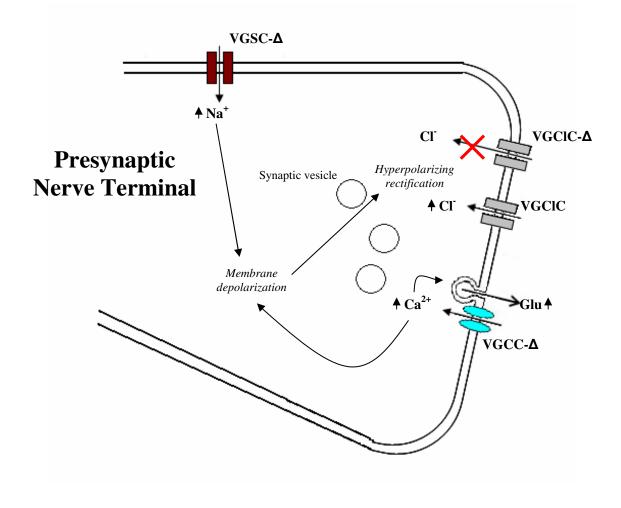
Figure 11: Mechanism by which L-glutamate release from presynaptic nerve terminals is increased by the presence of deltamethrin. An open triangle (Δ) indicates that respective channel has been modified by deltamethrin.

rectification of VGClCs cannot take place and the membrane remains depolarized. The subsequent increase in localized calcium influx at active release zones allow synaptic vesicles containing neurotransmitter to fuse with the presynaptic release machinery and release neurotransmitters into the synaptic cleft. Neurotransmitter release is prolonged and results in a more-than-additive response seen with certain binary mixtures of pyrethroids.

A second mechanism by which pyrethroids may elicit a more-than-additive response on L-glutamate release is by suppressing the activity of CaClCs, which are indirectly regulated by the metabotropic L-glutamate receptor (mGluR), part of the G-protein coupled receptor (GPCR) (Fig. 13).

Initially, the binding of released L-glutamate to the mGluRs associated with the synaptolemma of the presynaptic nerve terminal results in a conformational change of the GPCR activating the G-protein by binding GTP to the G_α subunit. Upon activation, G_α and $G_{\beta\gamma}$ subunit disassociation (due to activation of the complex by GTP) allows $G_{\beta\gamma}$ to bind directly to two regions on the VGCC, the α_1 subunit interaction domain (AID) and a sequence downstream in domain II (D2), ultimately inhibiting VGCC activity (De Waard, M. 1997; Symington, S.B. 1999) and decreasing neurotransmitter release. GTP is then hydrolyzed to GDP since this subunit possesses GTPase activity, allowing G_α to eventually reassociate with the $G_{\beta\gamma}$ complex.

The $G_{\beta\gamma}$ complex has also been implicated in the upregulation of PLC activity, as demonstrated by Symington *et al.* in *Paramecium tetraurelia* (Symington, S.B. 1999). PLC activity in calcium channel containing vesicles was further increased in the presence of deltamethrin (1 x 10^{-7} M), suggesting that deltamethrin enhances the $G_{\beta\gamma}$
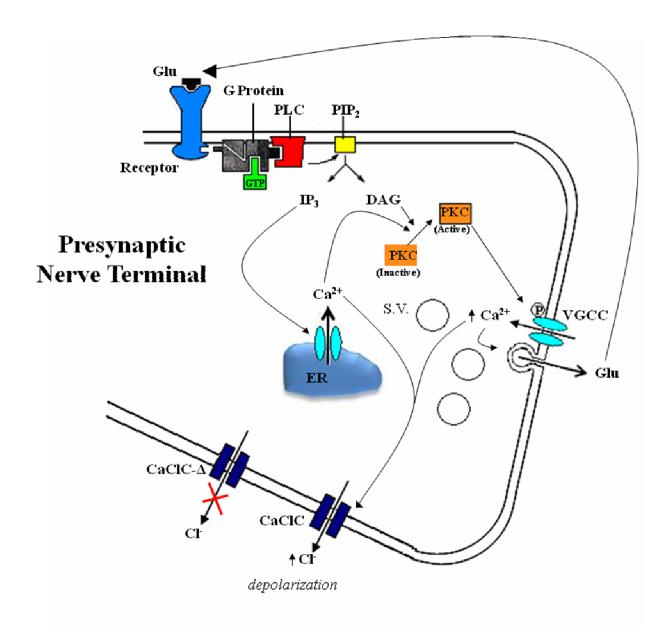
Figure 12: Mechanism by which intracellular signaling pathways increase L-glutamate release from presynaptic nerve terminals in the presence of deltamethrin. An open triangle (Δ) indicates that respective channel has been modified by deltamethrin.

activation of PLC (Symington, S.B. 1999) and these findings are consistent with the second mechanism by which pyrethroids may elicit a more-than-additive response on L-glutamate release.

Upon sustained stimulation of GPCR by excess L-glutamate, however, the G-protein complex can stimulate phospholipase C (PLC) to hydrolyze phosphatidyl insositol-biphosphate (PIP2) (Iglesias, I. 2007) to yield diacylglycerol (DAG) and inositol triphosphate (IP_3). IP_3 diffuses through the cytosol and promotes release of calcium from intracellular stores in the endoplasmic reticulum (ER). The mobilization of intracellular calcium in the presence of DAG then activates PKC (Cuellar, J.C. 2005). Upon PKC activation, VGCCs in the active release zone of presynaptic nerve terminals become phosphorylated and allow an increase influx of extracellular calcium, which in turn activates neurotransmitter release. The IP_3-dependent calcium released from intracellular stores is also available to activate CaClCs, allowing chloride ions to flux out, which depolarizes the membrane. If the membrane potential becomes more positive than the threshold values for VGSC and VGCC activation, these channels become inactivated, ultimately contributing to presynaptic inhibition (Graham, B. 1994; Meir, A. 1999). If CaClCs are blocked by pyrethroids, however, the synaptolemma will not be depolarized and VGSC and VGCC will remain activated allowing the increased release of L-glutamate, contributing to the more-than-additive response as seen with our pyrethroid binary mixtures.

Overall, the implications from this research suggest that the more-than-additive response binary mixtures previously determined by Frisbie (Frisbie, R.K. 2006) (deltamethrin plus *S*-bioallethrin, deltamethrin plus β-cyfluthrin, deltamethrin plus

cypermethrin, and deltamethrin plus fenpropathrin) may present a cumulative risk to mammals since three of the four elicited the more-than-additive response at pyrethroid concentrations less than those needed to elicit salivation and choreoathetosis (Rickard, J. and Brodie, M.E. 1985). Rickard and Brodie determined that 0.118 nmole deltamethrin / g brain tissue were needed to elicit salivation whereas 0.379 nmole deltamethrin / g brain tissue were needed to elicit choreoathetosis in rats. In the binary mixtures of pyrethroids tested, only deltamethrin plus *S*-bioallethrin exceeded these levels. Thus deltamethrin in binary mixture with β-cyfluthrin, cypermethrin, and fenpropathrin, however, are toxicologically relevant since these mixtures elicited the more-than-additive response at concentrations below the levels observed to elicit salivation and choreoathetosis and their actions likely contribute to the production of the neurotoxic symptoms of poisoning.

Our findings suggest that ClC-2 and/or CaClCs are potential target sites for certain pyrethroids and likely are important in pyrethroid neurotoxicity. To determine if one or both of these channels are modified by pyrethroids, the next line of investigation would entail heterologous expression of these channels from rat brain in *Xenopus laevis* oocytes using two-electrode voltage clamp to monitor chloride conductance in the presence and absence of selected pyrethroids. Since ClC-2 rectify membrane potential, it would be expected that depolarizing the oocyte during a voltage-clamp experiment (raising the membrane potential above E_{Cl}) would cause an inward flux of chloride ions. In the presence of our most potent pyrethroid, deltamethrin, it would be expected that chloride channel activity would be inhibited and peak current should be abolished. In the presence of a pyrethroid that does not modify VGClCs (e.g. tefluthrin), peak current should not be affected.

91

Heterologous expression of CaClCs would be expected to cause an outward flux of ions to resulting in a depolarization of the presynaptic nerve terminal and ultimately inactivating endogenous VGSCs and VGCCs. In the presence of deltamethrin, these channels would not inactivate VGSCs and VGCCs, resulting in a sustained depolarization of the membrane. Tefluthrin should not affect the gating kinetics of CaClC. Findings from these experiments would be expected to reveal whether or not certain pyrethroids modify these channels directly and if they could contribute to the more-than-additive response on L-glutamate release seen using our synaptosomal preparations.

APPENDIX A

CHLORIDE CHANNEL ANTAGONIST STRUCTURES

9-AC

DNDS

NPPB

Picrotoxinin

Met-Cys-Met-Pro-Cys-Phe-Thr-Thr-Asp-
His-Gln-Met-Ala-Arg-Lys-Cys-Asp-Asp-
Cys-Cys-Gly-Gly-Lys-Gly-Arg-Gly-Lys-
Cys-Tyr-Gly-Pro-Gln-Cys-Leu-Cys-Arg

rChlorotoxin

Appendix A. The structural configurations of the chloride channel blockers used in this study.

APPENDIX B

PYRETHROID STRUCTURES

Cypermethrin

Deltamethrin

Fenpropathrin

Tefluthrin

Appendix B. The structural configurations of the pyrethroids used in this study.

REFERENCES

Abalis, I. M., Eldefrawi, M. E., & Eldefrawi, A. T. (1986). Effects of insecticides on GABA-induced chloride influx into rat brain microsacs. *J. Toxicol. and Env. Health, 18*(1), 13-23.

Abdullaev, I. F., Rudkouskaya, A., Schools, G. P., Kimelberg, H. K., & Mongin, A. A. (2006). Pharmacological comparison of swelling-activated excitatory amino acid release and Cl⁻ currents in cultured rat astrocytes. *J. Physiol., 572*(Pt 3), 677-689.

Aldridge, W. N., B. Clothier, P. Forshaw, M.K. Johnson, V.H. Parker, R.J. Price, *et al.* (1978). The effect of DDT and the pyrethroids cismethrin and decamethrin on the acetylcholine and cyclic nucleotide content of rat brain. *Biochem. Pharmacol., 27*, 1703-1706.

Barnes, J. M., & Verschoyle, R. D. (1974). Toxicity of new pyrethroid insecticide. *Nature, 248*(450), 711.

Betz, H. (1990). Ligand-gated ion channels in the brain: The amino acid receptor superfamily. *Neuron, 5*(4), 383-392.

Bloomquist, J. R. (2003). Chloride channels as tools for developing selective insecticides. *Arch. In. Biochem. and Physiol., 54*(4), 145-156.

Bloomquist, J. R., Adams, P. M., & Soderlund, D. M. (1986). Inhibition of gamma-aminobutyric acid-stimulated chloride flux in mouse brain vesicles by polychlorocycloalkane and pyrethroid insecticides. *Neurotoxic., 7*(3), 11-20.

Browning, M. D., Bureau, M., Dudek, E. M., & Olsen, R. W. (1990). Protein kinase C and cAMP-dependent protein kinase phosphorylate the beta subunit of the purified gamma-aminobutyric acid A receptor. *Proc. Nat. Acad. Sci. U.S.A., 87*(4), 1315-1318.

Burke, S. P., Adams, M. E., & Taylor, C. P. (1993). Inhibition of endogenous glutamate release from hippocampal tissue by Ca^{2+} channel toxins. *E. J. Pharmacol., 238*(2-3), 383-386.

Burr, S. A., & Ray, D. E. (2004). Structure-activity and interaction effects of 14 different pyrethroids on voltage-gated chloride ion channels. *Tox. Sci.: J. Soc.Tox, 77*(2), 341-346.

Camacho, M., Machado, J. D., Montesinos, M. S., Criado, M., & Borges, R. (2006). Intragranular pH rapidly modulates exocytosis in adrenal chromaffin cells. *J. Neurochem., 96*(2), 324-334.

Casida, J. E., & Lawrence, L. J. (1985). Structure-activity correlations for interactions of bicyclophosphorus esters and some polychlorocycloalkane and pyrethroid insecticides with the brain-specific t-butylbicyclophosphorothionate receptor. *Env. Health Pers., 61*, 123-132.

Casida, J. E., & Quistad, G. B. (1998). Golden age of insecticide research: Past, present, or future? *Ann. Rev. Ent., 43*, 1-16.

Catterall, W. A. (1997). Modulation of sodium and calcium channels by protein phosphorylation and G proteins. *Adv. in Sec. Mess. and Phospho. Res., 31*, 159-181.

Catterall, W. A. (1998). Structure and function of neuronal Ca^{2+} channels and their role in neurotransmitter release. *Cell Calc., 24*(5-6), 307-323.

Catterall, W. A. (2000). Structure and regulation of voltage-gated Ca^{2+} channels. *Ann. Rev. Cell and Deve. Bio., 16*, 521-555.

Catterall, W. A., Striessnig, J., Snutch, T. P., Perez-Reyes, E., & International Union of Pharmacology. (2003). International union of pharmacology. XL. Compendium of voltage-gated ion channels: Calcium channels. *Pharmacol. Rev., 55*(4), 579-581.

Chakrapani, S., Bailey, T. D., & Auerbach, A. (2004). Gating dynamics of the acetylcholine receptor extracellular domain. *J. Gen. Physiol., 123*(4), 341-356.

Chinn, K., & Narahashi, T. (1986). Stabilization of sodium channel states by deltamethrin in mouse neuroblastoma cells. *J. Physiol., 380*, 191-207.

Choi, J. S., & Soderlund, D. M. (2006). Structure-activity relationships for the action of 11 pyrethroid insecticides on rat $Na_V1.8$ sodium channels expressed in xenopus oocytes. *Toxic. and App. Pharmacol., 211*(3), 233-244.

Clark, J. M. (1994). In L.W. Chang, A.R.S. Dyer (Eds.), <u>*Handbook of neurotoxicology*</u> [Effects and Mechanisms of Action of Pyrethrin and Pyrethroid insecticides] . New York, Basel, Hong Kong: Marcel Dekker Inc.

Clark, J. M. (1995). In J.M. Clark (Ed.), <u>*Molecular action of insecticides on ion channels*</u> [Ion-channel diversity]. Washington D.C.: American Chemical Society.

Clark, J. M., & S.B. Symington. (2008). *Neurotoxic implications of the agonistic action of CS-syndrome pyrethroids on the N-type $Ca_V2.2$ calcium channel.* Unpublished manuscript.

Clark, J. M., & Symington, S. B. (2007). Pyrethroid action on calcium channels: Neurotoxicological implications. *Invertebrate Neurosci., 7*(1), 3-16.

Clark, S., Jordt, S. E., Jentsch, T. J., & Mathie, A. (1998). Characterization of the hyperpolarization-activated chloride current in dissociated rat sympathetic neurons. *J. Physiol., 506* (Pt 3), 665-678.

Cuellar, J. C., Griffith, E. L., & Merlin, L. R. (2005). Contrasting roles of protein kinase C in induction versus suppression of group I mGluR-mediated epileptogenesis in vitro. *J. Neurophys., 94*(5), 3643-3647.

Dalton, S., Gerzanich, V., Chen, M., Dong, Y., Shuba, Y., & Simard, J. M. (2003). Chlorotoxin-sensitive Ca^{2+}-activated Cl^- channel in type R2 reactive astrocytes from adult rat brain. *Glia, 42*(4), 325-339.

De Castro, F., Geijo-Barrientos, E., & Gallego, R. (1997). Calcium-activated chloride current in normal mouse sympathetic ganglion cells. *J. Physiol., 498 (Pt 2)*(Pt 2), 397-408.

De Waard, M., Liu, H., Walker, D., Scott, V. E., Gurnett, C. A., & Campbell, K. P. (1997). Direct binding of G-protein betagamma complex to voltage-dependent calcium channels. *Nature, 385*(6615), 446-450.

Dinudom, A., Young, J. A., & Cook, D. I. (1993). Na^+ and Cl^- conductances are controlled by cytosolic Cl^- concentration in the intralobular duct cells of mouse mandibular glands. *J. Membr. Biol., 135*(3), 289-295.

Eggermont, J. (2004). Calcium-activated chloride channels: (un)known, (un)loved? *Proc. Am. Thor. Soc., 1*(1), 22-27.

Elliott, M., A.W. Farnham, N.F. Janes, P.H. Needham, D.A. Pulman. (1974). Insecticidally active conformations of pyrethroids. In G. K. Kohn (Ed.), *Mechanism of pesticide action* (pp. 80-91). Washington, D.C.: American Chemical Society.

Enan, E. and Matsumura, F. (1991). Stimulation of protein phosphorylation in intact rat brain synaptosomes by a pyrethroid insecticide, deltamethrin. *Pest. Biochem. Physiol., 39*, 182.

Evans, R. M., & Zamponi, G. W. (2006). Presynaptic Ca^{2+} channels--integration centers for neuronal signaling pathways. *Trends in Neurosci., 29*(11), 617-624.

Forshaw, P. J., Lister, T., & Ray, D. E. (1993). Inhibition of a neuronal voltage-dependent chloride channel by the type II pyrethroid, deltamethrin. *Neuropharm., 32*(2), 105-111.

Forshaw, P. J., Lister, T., & Ray, D. E. (2000). The role of voltage-gated chloride channels in type II pyrethroid insecticide poisoning. *Toxicol. and App. Pharmacol., 163*(1), 1-8.

Forshaw, P. J., & Ray, D. E. (1990). A novel action of deltamethrin on membrane resistance in mammalian skeletal muscle and non-myelinated nerve fibres. *Neuropharm., 29*(1), 71-81.

Frisbie, R. K. (2006). Actions of pyrethroids alone and in binary mixtures at presynaptic nerve terminals from rat brain. (M.S., University of Massachusetts-Amherst).

Furukawa, T., Ogura, T., Katayama, Y., & Hiraoka, M. (1998). Characteristics of rabbit ClC-2 current expressed in xenopus oocytes and its contribution to volume regulation. *Am. J. Physiol., 274*(2 Pt 1), C500-12.

Gammon, D. W., M.A. Brown, & J.E. Casida. (1981). Two classes of pyrethroid action in the cockroach. *Pest. Biochem. Physiol., 15*, 181-191.

Gammon, D. W., Lawrence, L. J., & Casida, J. E. (1982). Pyrethroid toxicology: Protective effects of diazepam and phenobarbital in the mouse and the cockroach. *Tox. and App. Pharm., 66*(2), 290-296.

Graham, B., & Redman, S. (1994). A simulation of action potentials in synaptic boutons during presynaptic inhibition. *J. Neurophysiol., 71*(2), 538-549.

Grunder, S., Thiemann, A., Pusch, M., & Jentsch, T. J. (1992). Regions involved in the opening of CIC-2 chloride channel by voltage and cell volume. *Nature, 360*(6406), 759-762.

Hartzell, C., Putzier, I., & Arreola, J. (2005). Calcium-activated chloride channels. *Ann. Rev. Physiol., 67*, 719-758.

Heuschneider, G., & Schwartz, R. D. (1989). cAMP and forskolin decrease gamma-aminobutyric acid-gated chloride flux in rat brain synaptoneurosomes. *Proc. Nat. Acad. Sci. U.S.A., 86*(8), 2938-2942.

Hildebrand, M. E., McRory, J. E., Snutch, T. P., & Stea, A. (2004). Mammalian voltage-gated calcium channels are potently blocked by the pyrethroid insecticide allethrin. *J. Pharmacol. Exp. Ther., 308*(3), 805-813.

Hille, B. (2001). *Ion channels of excitable membranes* (third ed.). Sunderland, MA: Sinauer Associates, Inc.

Hossain, M. M., Suzuki, T., Sato, I., Takewaki, T., Suzuki, K., & Kobayashi, H. (2004). The modulatory effect of pyrethroids on acetylcholine release in the hippocampus of freely moving rats. *Neurotox., 25*(5), 825-833.

Hossain, M. M., Suzuki, T., Unno, T., Komori, S., & Kobayashi, H. (2008). Differential presynaptic actions of pyrethroid insecticides on glutamatergic and GABAergic neurons in the hippocampus. *Toxicol., 243*(1-2), 155-163.

Iglesias, I., Castillo, C. A., Leon, D., Ruiz, M. A., Albasanz, J. L., & Martin, M. (2007). Metabotropic glutamate receptor/phospholipase C system in female rat heart. *Brain Res., 1153*, 1-11.

Jackson, M. B. (1995). Presynaptic excitability. *Int. Rev. Neurobiol., 38*, 201-251.

Jang, I. S., Nakamura, M., Ito, Y., & Akaike, N. (2006). Presynaptic GABA$_A$ receptors facilitate spontaneous glutamate release from presynaptic terminals on mechanically dissociated rat CA3 pyramidal neurons. *Neurosci., 138*(1), 25-35.

Jarvis, S. E., & Zamponi, G. W. (2001). Interactions between presynaptic Ca^{2+} channels, cytoplasmic messengers and proteins of the synaptic vesicle release complex. *Trends in Pharmacol. Sci., 22*(10), 519-525.

Jentsch, T. J., & Gunther, W. (1997). Chloride channels: An emerging molecular picture. *BioEssays : News and Rev. in Molec., Cell. and Dev. Biol., 19*(2), 117-126.

Jentsch, T. J., Poet, M., Fuhrmann, J. C., & Zdebik, A. A. (2005). Physiological functions of ClC Cl$^-$ channels gleaned from human genetic disease and mouse models. *Ann.Rev. Physiol., 67*, 779-807.

Jentsch, T. J., Pusch, M., Rehfeldt, A., & Steinmeyer, K. (1993). The ClC family of voltage-gated chloride channels: Structure and function. *Ann. N.Y. Acad. Sci., 707*, 285-293.

Jentsch, T. J., Stein, V., Weinreich, F., & Zdebik, A. A. (2002). Molecular structure and physiological function of chloride channels. *Physiol. Rev., 82*(2), 503-568.

Jordt, S. E., & Jentsch, T. J. (1997). Molecular dissection of gating in the ClC-2 chloride channel. *EMBO Journal, 16*(7), 1582-1592.

Kash, T. L., Kim, T., Trudell, J. R., & Harrison, N. L. (2004). Evaluation of a proposed mechanism of ligand-gated ion channel activation in the GABA$_A$ and glycine receptors. *Neurosci.Lett., 371*(2-3), 230-234.

Lawrence, L. J., & J.E. Casida. (1982). Pyrethroid toxicology: Mouse intracerebral structure-toxicity relationships. *Pest. Biochem. Physiol., 18*, 9-14.

Lawrence, L. J., & Casida, J. E. (1983). Stereospecific action of pyrethroid insecticides on the gamma-aminobutyric acid receptor-ionophore complex. *Science (New York, N.Y.), 221*(4618), 1399-1401.

Leeb-Lundberg, F., R.W. Olsen. (1980). Picrotoxinin binding as a probe of the GABA postsynaptic membrane receptor—ionophore complex. In Yamamura, H.I., R.W. Olsen and E. Usdin (Ed.), *Psychopharm. and Bio. Neuro. Rec.* (pp. 593–606). New York: Elsevier.

Leidenheimer, N. J., McQuilkin, S. J., Hahner, L. D., Whiting, P., & Harris, R. A. (1992). Activation of protein kinase C selectively inhibits the gamma-aminobutyric acidA receptor: Role of desensitization. *Molec. Pharmacol., 41*(6), 1116-1123.

Lorenzon, N. M., & Beam, K. G. (2000). Calcium channelopathies. *Kid. Inter., 57*(3), 794-802.

Lund, A. E., & T. Narahashi. (1983). Kinetics of sodium channel modification as the basis for the variation in the nerve membrane effects of pyrethroids and DDT analogs. *Pest. Biochem. Physiol., 20*, 203-216.

Macdonald, R. L., & Olsen, R. W. (1994). GABA$_A$ receptor channels. *Ann. Rev. Neurosci., 17*, 569-602.

Martinez-Pinna, J., McLachlan, E. M., & Gallego, R. (2000). Distinct mechanisms for activation of Cl$^-$ and K$^+$ currents by Ca^{2+} from different sources in mouse sympathetic neurones. *J. Physiol., 527 Pt 2*, 249-264.

Matsumura, F., Clark, J. M., & Matsumura, F. M. (1989). Deltamethrin causes changes in protein phosphorylation activities associated with post-depolarization events in the synaptosomes from the optic lobe of squid, loligo pealei. *Comp. Biochem. and Physiol..C, Comp. Pharmacol. and Toxicol., 94*(2), 381-390.

Meir, A., Ginsburg, S., Butkevich, A., Kachalsky, S. G., Kaiserman, I., Ahdut, R., *et al*. (1999). Ion channels in presynaptic nerve terminals and control of transmitter release. *Physiol. Rev., 79*(3), 1019-1088.

Narahashi, T., & Yamasaki, T. (1960). Mechanism of increase in negative after-potential by dicophanum (DDT) in the giant axons of the cockroach. *J. Physiol., 152*, 122-140.

Narahashi, T. (1992). Nerve membrane Na$^+$ channels as targets of insecticides. *Trends in Pharmacol. Sci., 13*(6), 236-241.

Nicholls, D. G., Sihra, T. S., & Sanchez-Prieto, J. (1987). Calcium-dependent and -independent release of glutamate from synaptosomes monitored by continuous fluorometry. *J. Neurochem., 49*(1), 50-57.

Nilius, B., & Droogmans, G. (2003). Amazing chloride channels: An overview. *Acta Physiologica Scandinavica, 177*(2), 119-147.

Nilius, B., Eggermont, J., Voets, T., & Droogmans, G. (1996). Volume-activated Cl$^-$ channels. *Gen. Pharmacol., 27*(7), 1131-1140.

Okada, Y., Fujiyama, R., Miyamoto, T., & Sato, T. (2000). Comparison of a Ca^{2+}-gated conductance and a second-messenger-gated conductance in rat olfactory neurons. *J. Exp. Biol., 203*(Pt 3), 567-573.

Oosawa, Y., & Yamagishi, S. (1989). Rat brain glutamate receptors activate chloride channels in xenopus oocytes coupled by inositol trisphosphate and Ca^{2+}. *J. Physiol., 408*, 223-232.

Park, K., Begenisich, T., & Melvin, J. E. (2001). Protein kinase A activation phosphorylates the rat ClC-2 Cl^- channel but does not change activity. *J. Membr. Biol., 182*(1), 31-37.

Perez-Reyes, E. (2003). Molecular physiology of low-voltage-activated t-type calcium channels. *Physiol. Rev., 83*(1), 117-161.

Porter, N. M., Twyman, R. E., Uhler, M. D., & Macdonald, R. L. (1990). Cyclic AMP-dependent protein kinase decreases $GABA_A$ receptor current in mouse spinal neurons. *Neuron, 5*(6), 789-796.

Potier, B., Dutar, P., & Lamour, Y. (1993). Different effects of omega-conotoxin GVIA at excitatory and inhibitory synapses in rat CA1 hippocampal neurons. *Brain Res., 616*(1-2), 236-241.

Pusch, M., Zifarelli, G., Murgia, A. R., Picollo, A., & Babini, E. (2006). Channel or transporter? the CLC saga continues. *Exper. Physiol., 91*(1), 149-152.

Ray, D. E., & J.E. Cremer. (1979). The action of decamethrin (a synthetic pyrethroid) on the rat. *Pest. Biochem. Physiol., 10*, 333-340.

Ray, D. E., & J.R. Fry. (2006). A reassessment of the neurotoxicity of pyrethroid insecticides. *Pharm. and Therap., 111*, 174-193.

Ray, D. E., S. Sutharson, & P.J. Forshaw. (1997). Actions of pyrethroid insecticides on voltage-gated chloride channels in neuroblastoma cells. *Neurtox., 18*(3), 755-760.

Rickard, J. and Brodie, M.E. (1985). Correlation of blood brain levels of the neurotoxic pyrethroid deltamethrin with the onset of symptoms in rats. *Pest. Biochem. Physiol., 23*, 143-156.

Rudomin, P. (1990). Presynaptic inhibition of muscle spindle and tendon organ afferents in the mammalian spinal cord. *Trends in Neurosci., 13*(12), 499-505.

Shafer, T. J., Meyer, D. A., & Crofton, K. M. (2005). Developmental neurotoxicity of pyrethroid insecticides: Critical review and future research needs. *Envir. Health Persp., 113*(2), 123-136.

Smith, R. L., Clayton, G. H., Wilcox, C. L., Escudero, K. W., & Staley, K. J. (1995). Differential expression of an inwardly rectifying chloride conductance in rat brain neurons: A potential mechanism for cell-specific modulation of postsynaptic inhibition. *J. Neurosci.: Off. J. Soc. Neurosci., 15*(5 Pt 2), 4057-4067.

Soderlund, D. M., & Bloomquist, J. R. (1989). Neurotoxic actions of pyrethroid insecticides. *Ann. Rev. Entomol., 34*, 77-96.

Soderlund, D. M., Clark, J. M., Sheets, L. P., Mullin, L. S., Piccirillo, V. J., Sargent, D., *et al*. (2002). Mechanisms of pyrethroid neurotoxicity: Implications for cumulative risk assessment. *Toxicol., 171*(1), 3-59.

Soderlund, D. M., Smith, T. J., & Lee, S. H. (2000). Differential sensitivity of sodium channel isoforms and sequence variants to pyrethroid insecticides. *Neurotox., 21*(1-2), 127-137.

Staley, K. (1994). The role of an inwardly rectifying chloride conductance in postsynaptic inhibition. *J. Neurophys., 72*(1), 273-284.

Suzuki, M., Morita, T., & Iwamoto, T. (2006). Diversity of Cl^- channels. *Cell. Molec. Life Sci.: CMLS, 63*(1), 12-24.

Symington, S. B. (2005). The action of T- and CS-syndrome pyrethroids on voltage-sensitive calcium channels in rat brain. (Ph.D., University of Massachusetts Amherst).

Symington, S. B., & J.M. Clark. (2005). Action of deltamethrin on N-type ($Ca_V2.2$) voltage-sensitive calcium channels in rat brain. *Pest. Biochem. Physiol., 82*, 1-15.

Symington, S. B., R.K. Frisbie, H-J. Kim, & J.M. Clark. (2007a). Mutation of threonine 422 to glutamic acid mimics the phosphorylation state and alters the action of deltamethrin on $Ca_V2.2$. *Pest. Biochem. Physiol., 88*(3), 312-320.

Symington, S. B., R.K. Frisbie, K.D. Lu, & J.M Clark. (2007b). Action of cismethrin and deltamethrin on functional attributes of isolated presynaptic nerve terminals from rat brain. *Pest. Biochem. Physiol., 87*, 172-181.

Symington, S. B., Zhang, A., & Clark, J. M. (1999). The action of pyrethroids on the voltage-sensitive calcium channel of *paramecium tetraurelia*. *Pest. Sci., 55*, 1027-1040.

Takken, W. (2002). Do insecticide-treated bednets have an effect on malaria vectors? *Trop. Med. Int. Health, 7*, 1022-1030.

Tedford, H. W., & Zamponi, G. W. (2006). Direct G protein modulation of Ca_V2 calcium channels. *Pharmacol. Rev., 58*(4), 837-862.

Thiemann, A., Grunder, S., Pusch, M., & Jentsch, T. J. (1992). A chloride channel widely expressed in epithelial and non-epithelial cells. *Nature, 356*(6364), 57-60.

Thompson, C. H., Fields, D. M., Olivetti, P. R., Fuller, M. D., Zhang, Z. R., Kubanek, J., *et al*. (2005). Inhibition of ClC-2 chloride channels by a peptide component or components of scorpion venom. *J. Membr. Biol., 208*(1), 65-76.

Trainer, V. L., McPhee, J. C., Boutelet-Bochan, H., Baker, C., Scheuer, T., Babin, D., *et al*. (1997). High affinity binding of pyrethroids to the alpha subunit of brain sodium channels. *Molec. Pharmacol., 51*(4), 651-657.

United States Environmental Protection Agency. (2006). *Organophosphate cumulative risk assessment; notice of availability (EPA-HQ-OPP-2006-0618-0001)*

United States Environmental Protection Agency. (2007a). *Accomplishments under the food quality protection act (FQPA).*, 2007, from http://www.epa.gov/pesticides/regulating/laws/fqpa/fqpa_accomplishments.htm

United States Environmental Protection Agency. (2007b). *Assessing pesticide cumulative risk.*, 2007, from http://www.epa.gov/pesticides/cumulative/

United States Environmental Protection Agency. (2007c). *The food quality protection act (FQPA) background.*, 2007, from http://www.epa.gov/pesticides/regulating/laws/fqpa/backgrnd.htm

United States Environmental Protection Agency. (2007d). *N-methyl carbamate revised cumulative risk assessment; notice of availability (EPA-HQ-OPP-2007-0935-0001)*

Vautrin, J., Schaffner, A. E., & Barker, J. L. (1994). Fast presynaptic GABA$_A$ receptor-mediated Cl$^-$ conductance in cultured rat hippocampal neurones. *J. Physiol., 479* (Pt 1), 53-63.

Verschoyle, R.D. and J.M. Barnes. (1972). Toxicity of natural and synthetic pyrethrins to rats. *Pest. Biochem. Physiol., 2*, 308-311.

Verschoyle, R. D., & Aldridge, W. N. (1980). Structure-activity relationships of some pyrethroids in rats. *Arch. Tox., 45*(4), 325-329.

Weinreich, F., & Jentsch, T. J. (2001). Pores formed by single subunits in mixed dimers of different ClC chloride channels. *J. Biol. Chem., 276*(4), 2347-2353.

Wennemuth, G., Westenbroek, R. E., Xu, T., Hille, B., & Babcock, D. F. (2000). Ca$_V$2.2 and Ca$_V$2.3 (N- and R-type) Ca^{2+} channels in depolarization-evoked entry of Ca^{2+} into mouse sperm. *J. Biol. Chem., 275*(28), 21210-21217.

Wheeler, D. B., Randall, A., & Tsien, R. W. (1994). Roles of N-type and Q-type Ca^{2+} channels in supporting hippocampal synaptic transmission. *Science (New York, N.Y.), 264*(5155), 107-111.

Wright, C.D.P., P.J. Forshaw, D.E. Ray. (1988). Classification of the actions of ten pyrethroid insecticides in the rat, using the trigeminal reflex and skeletal muscle as test systems. *Pest. Biochem. Physiol., 90*, 79-86.

Zamponi, G. W., Bourinet, E., Nelson, D., Nargeot, J., & Snutch, T. P. (1997). Crosstalk between G proteins and protein kinase C mediated by the calcium channel alpha1 subunit. *Nature, 385*(6615), 442-446.

Zhang, S. J., & Jackson, M. B. (1993). GABA-activated chloride channels in secretory nerve endings. *Science (New York, N.Y.), 259*(5094), 531-534.