Effect of Pyrethroid Insecticides on Gene Expression in the Mammalian Central Nervous System

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“A dissertation submitted to the faculty of the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of doctor of philosophy in the Curriculum in Toxicology.”

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Abstract

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(Under the direction of Kevin M. Crofton, Ph.D.)

Pyrethroids interfere with nervous system function by increasing neuronal excitability. Increased excitability underlies the toxicity observed at the whole organism level following an acute pyrethroid exposure. However, changes in neuronal excitability also trigger de novo gene expression which may impact neuronal function. This aspect of pyrethroid toxicity has not been extensively examined. The present studies test the hypothesis that in vivo pyrethroid exposures, at doses surrounding the threshold for neurobehavioral effects, result in changes in gene expression in the rat cortex. In the first aim, adult rats were orally dosed with deltamethrin (DLT: 0.3, 1, 3 mg/kg), permethrin (PERM: 1, 10, 100 mg/kg) or vehicle. Frontal cortex was collected at 6 hr and global transcriptional profiles were generated. Dose-dependent changes in gene expression were identified using penalized linear and isotonic regressions. A set of altered transcripts were then confirmed by qRT-PCR. In addition, rats were dosed with either DLT (3 mg/kg), PERM (100 mg/kg) or vehicle and cortical tissue collected at 1, 3, 6 and 9 hr. Expression of transcripts examined by qRT-PCR in the dose-response studies were investigated in this time course cohort. Functional category analysis identified ‘branching morphogenesis’ as a biological process potentially sensitive to pyrethroids. This prediction was confirmed in an
in vitro model of neuronal morphogenesis. The time course of expression for Camk1g was further examined by qRT-PCR and Western blot. Expression of the Camk1g1 mRNA splice variant changed in response to pyrethroids with no detectable change in Camk1g1 protein. The second aim determined if Type I and Type II pyrethroids produce similar effects on gene transcription in the cortex. Rats were dosed with vehicle or either a Type I or Type II pyrethroid at doses that produce the same effect on an apical behavior. Cortex was sampled at 3 and 6 hr and global transcriptional profiles were generated. Qualitatively similar but quantitatively different patterns of expression between Type I and Type II pyrethroids were observed that is consistent with increases in neuronal excitability. These data contribute to a comprehensive mode-of-action model for pyrethroids.
This work is dedicated to the whole of the Harrill family for their love, support and advice for which my everlasting love and gratitude is due:

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And to Ms. Alison I. Hege, the constant source of joy and light in my life.
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Table of Contents

List of Tables ............................................................................................................................................ ix

List of Figures .................................................................................................................................................. x

List of Abbreviations ................................................................................................................................... xii

List of Symbols ............................................................................................................................................ xiii

Chapter 1: Effect of Pyrethroid Insecticides on Gene Expression in the Mammalian Central Nervous System ................................................................. 1

I. Overview ....................................................................................................................................................... 2

II. Pyrethroid usage ........................................................................................................................................... 3

III. Pyrethroid toxicity ...................................................................................................................................... 4

IV. Ion channel functions in the nervous system .................................................................................. 9

   IVa. Molecular mechanisms of neuronal firing and synaptic communication ........................................... 9

   IVb. Influence of neuronal activity on gene expression .............................................................................. 11

V. Molecular targets of pyrethroids ............................................................................................................ 13

   Va. Voltage-sensitive Na\(^+\) channels and pyrethroid effects on neuronal firing rates ............................... 13

   Vb. Pyrethroid effects on voltage-sensitive Ca\(^{2+}\) channels ................................................................. 16

   Vc. Pyrethroid effects on voltage-sensitive Cl\(^-\) channels .................................................................. 19

   Vd. Pyrethroid effects on GABAergic receptors .................................................................................... 20

VI. Pyrethroid effects on gene expression ............................................................................................. 21

VII. Hypothesis and goals .......................................................................................................................... 24
Chapter 4: Transcriptional Response of Rat Frontal Cortex to Acute Pyrethroid Exposure................................................................. 141

Abstract........................................................................................................ 142

Introduction .................................................................................................... 143

Methods........................................................................................................... 145

Results............................................................................................................ 152

Discussion ..................................................................................................... 158

Funding ......................................................................................................... 168

Acknowledgements .................................................................................... 168

Works Cited ................................................................................................ 181

Chapter 5: Discussion and Future Directions ................................................... 189

Works Cited ................................................................................................ 197

Appendix A. Chapter 2 Supplementary Material ........................................... 202

Appendix B. Chapter 4 Supplementary Material........................................... 206
List of Tables

Table 2.1. Group sizes of cohorts used in this study. ............................................................ 80
Table 2.2. List of probe sets with dose-dependent changes in expression for deltamethrin.. 81
Table 2.3. List of probe sets with dose-dependent changes in expression for permethrin. ... 84
Table 2.4. qRT-PCR confirmation of transcripts identified as dose-responsive. ................. 86
Table 2.5. Two-way analysis of variance (ANOVA) for qRT-PCR time course data. ......... 87
Table 2.6. Significant Analysis of Function and Expression (SAFE). ................................. 88
Table 2.7. Pharmacokinetic estimates of pyrethroid brain concentrations. ......................... 89

Table 3.1. Camk1g and Camk1g splice variant specific TaqMan assays............................. 128
Table 3.2. Statistical analysis of qRT-PCR dose-response data. ...................................... 129
Table 3.3. Statistical analysis of qRT-PCR time course data ............................................ 130

Table 4.1. Enrichment scores for gene-of-interest lists derived from
Venn Diagram comparison. ................................................................................ 169
Table 4.2. Genes changed by Type II pyrethroid exposure. ............................................. 170

Appendix A, Table 1. Comparison of mean coefficients of variation
(CV) between GCOSv1.2 and RMA microarray expression
summaries. ....................................................................................................... 203

Appendix A, Table 2. Taqman® qRT-PCR assay information........................................ 204
Appendix B, Table 1. Taqman® qRT-PCR assay information........................................ 207
Appendix B, Table 2. Statistical Analysis of qRT-PCR Data ........................................ 208
List of Figures

Figure 1.1. Mode-of-action for the acute effects of pyrethroids...................................................... 28
Figure 1.2. Examples of pyrethroid chemical structures. ................................................................. 29
Figure 2.1. Comparison of PIR and SAM regression methods.......................................................... 90
Figure 2.2. Dose-response functions identified by PIR and SAM regression methods................. 91
Figure 2.3. Comparison of probe sets identified by PIR or SAM between pyrethroids................. 92
Figure 2.4. qRT-PCR time course results...................................................................................... 93
Figure 2.5. Composition and expression patterns of significantly enriched GO categories from SAFE analysis. ................................................................. 94
Figure 2.6. Pyrethroid effects on branching and neurite length in primary cortical cell cultures. ...................................................................................... 96
Figure 3.1. Exon schematic of rat Camk1g1 and Camk1g2 ................................................................. 131
Figure 3.2. Dose-response for Camk1g1 and Camk1g2 mRNA expression..................................... 132
Figure 3.3. Time course for Camk1g1 and Camk1g2 mRNA expression. ....................................... 133
Figure 3.4. Rat Camk1g1 and Camk1g2 protein sequences and Camk1g1 protein assay development ......................................................................................... 134
Figure 3.5. Camk1g1 protein expression following acute pyrethroid exposure. ......................... 135
Figure 4.1. Chemical structures of pyrethroids.............................................................................. 172
Figure 4.2. Pyrethroid effects on motor activity............................................................................ 173
Figure 4.3. Volcano plots of pair-wise comparisons of microarray data........................................ 174
Figure 4.4. Linear Models for Microarray Data (LIMMA) analysis scheme................................. 175
Figure 4.5. Results of LIMMA analysis for Type II pyrethroids..................................................... 176
Figure 4.6. Comparison of gene expression changes across compounds. .................................. 177
Figure 4.7. qRT-PCR results........................................................................................................ 178
Figure 4.8. Ingenuity® pathway analysis of genes commonly affected
by Type II pyrethroids. ................................................................. 179

Figure 4.9. Schematic of putative pyrethroid gene regulatory network. ....................... 180

Appendix A, Figure 1. Identification of dose-responsive probe sets 
by linear regression or penalized linear (SAM) regression. ......................... 205

Appendix B, Figure 1. Dimensional reduction of microarray data: 
effect of mean centering on measures of significance. ................................. 214
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>BIF</td>
<td>bifenthrin</td>
</tr>
<tr>
<td>CYF</td>
<td>cyfluthrin</td>
</tr>
<tr>
<td>CYP</td>
<td>cypermethrin</td>
</tr>
<tr>
<td>DLT</td>
<td>deltamethrin</td>
</tr>
<tr>
<td>EDL</td>
<td>Equipotent Dose Level</td>
</tr>
<tr>
<td>FDR</td>
<td>false discovery rate</td>
</tr>
<tr>
<td>GCOS</td>
<td>GeneChip® Operating Software</td>
</tr>
<tr>
<td>GO</td>
<td>Gene ontology</td>
</tr>
<tr>
<td>HPA</td>
<td>hypothalamic-pituitary-adrenal axis</td>
</tr>
<tr>
<td>IEG</td>
<td>immediate early gene</td>
</tr>
<tr>
<td>KEGG</td>
<td>Kyoto encyclopedia of genes and genomes</td>
</tr>
<tr>
<td>MAP</td>
<td>mitogen activated protein</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>PERM</td>
<td>permethrin</td>
</tr>
<tr>
<td>PIR</td>
<td>Penalized Isotonic Regression</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative Real-Time Polymerase Chain Reaction</td>
</tr>
<tr>
<td>RMA</td>
<td>Robust Multi-Array Analysis</td>
</tr>
<tr>
<td>SAFE</td>
<td>Significant Analysis of Function and Expression</td>
</tr>
<tr>
<td>SAM</td>
<td>Significant Analysis of Microarrays</td>
</tr>
<tr>
<td>TEF</td>
<td>tefluthrin</td>
</tr>
<tr>
<td>TRT</td>
<td>treatment</td>
</tr>
</tbody>
</table>
List of Symbols

Acpl2: acid phosphatase-like 2
Adipor: adiponectin receptor 2
Angpt14: angiopoietin-like 4
Arc: activity regulated cytoskeletal-associated protein
Arhgap7: deleted in liver cancer 1
Arrdc2: arrestin domain containing 2
Asah3l: N-acylsphingosine amidohydrolase 3-like
Asph: aspartate beta-hydroxylase
Axud1: AXIN1 up-regulated 1
B3galt3: UDP-Gal:betaGlcNAc beta 1,3-galactosyltransferase, polypeptide 3
B3galt5: UDP-Gal:betaGlcNAc beta 1,3-galactosyltransferase, polypeptide 5
Bag3: BCL2-associated athanogene 3
Bcat: branched chain aminotransferase 1, cytosolic
Bcl2l1: Bcl2-like 1
Bcl6: B-cell leukemia/lymphoma 6 (predicted)
Bdnf: brain derived neurotrophic factor
Bves: blood vessel epicardial substance
c-fos: FBJ murine osteosarcoma viral oncogene homolog
Cables1: Cdk5 and Abl enzyme substrate 1 (predicted)
Cacng2: calcium channel, voltage-dependent, gamma subunit 2
Camk1g: calcium/calmodulin-dependent protein kinase I gamma
Camk1g1: calcium/calmodulin-dependent protein kinase I gamma 1
Camk1g2: calcium/calmodulin-dependent protein kinase I gamma 2
Cbr3: carbonyl reductase 3 (predicted)
Cebpb: CCAAT/enhancer binding protein (C/EBP), beta
Cd163: CD163 antigen (predicted)
Cdc42ep4: CDC42 effector protein (Rho GTPase binding) 4 (predicted)
Cdkn1a: cyclin-dependent kinase inhibitor 1A
Chrm4: cholinergic receptor, muscarinic 4
Clec14a: C-type lectin domain family 14, member a
Cnksr3: Cnksr family member 3
Ctrl: chymotrypsin-like
Cttnb2nl: CTTNB2 N-terminal like (predicted)
Cxcr4: chemokine (C-X-C motif) receptor 4
CREB: cAMP responsive element binding protein
Crh: corticotropin releasing hormone
Cryab: crystallin, alpha B
Cxc112: chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1)
Ddc: dopa decarboxylase (aromatic L-amino acid decarboxylase)
Degs: degenerative spermatocyte homolog 1
Dnajb5: DnaJ (Hsp40) homolog, subfamily B, member 5 (predicted)
Dusp1: dual specificity phosphatase 1
Dusp5: dual specificity phosphatase 5
Dusp6: dual specificity phosphatase 6
Dyncl11: dynein cytoplasmic 1 intermediate chain 1
Egr1: early growth response 1
Egr2: early growth response 2
Ephb3: Eph receptor B3 (predicted)
ERK: extracellular regulated MAP kinase
Errfi1: ERBB receptor feedback inhibitor 1
Ets2: v-ets erythroblastosis virus E26 oncogene homolog 2
Fbxo22: F-box only protein 22
Finb: ras responsive element binding protein 1 (predicted)
Fkbp51: FK506 binding protein 5
Fmo2: flavin containing monooxygenase 2
Fmo3: flavin containing monooxygenase 3
Fst: follistatin
Gad45b: growth arrest and DNA-damage-inducible, beta
Gfpt2: glutamine-fructose-6-phosphate transaminase 2
Gjb6: gap junction protein, beta 6, 30kDa
Gna14: guanine nucleotide binding protein, alpha 14
Gpd1: glycerol-3-phosphate dehydrogenase 1
Heatr1: HEAT repeat containing 1 (predicted)
Hes1: hairy and enhancer of split 1
Homer1: homer homolog 1
Hs3st1: heparan sulfate (glucosamine) 3-O-sulfotransferase 1
Hsp27: heat shock 27kDa protein 1
Hyal2: hyaluronoglucosaminidase 2
<table>
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<tr>
<th>Symbol</th>
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<tbody>
<tr>
<td>Hyou1:</td>
<td>hypoxia up-regulated 1</td>
</tr>
<tr>
<td>Id1:</td>
<td>inhibitor of DNA binding 1</td>
</tr>
<tr>
<td>Ier2:</td>
<td>immediate early response 2</td>
</tr>
<tr>
<td>Ier5:</td>
<td>immediate early response 5</td>
</tr>
<tr>
<td>Ier5l:</td>
<td>immediate early response 5-like</td>
</tr>
<tr>
<td>Igfpb3:</td>
<td>insulin-like growth factor binding protein 3</td>
</tr>
<tr>
<td>Il6r:</td>
<td>interleukin 6 receptor</td>
</tr>
<tr>
<td>Irs2:</td>
<td>insulin receptor substrate 2</td>
</tr>
<tr>
<td>Junb:</td>
<td>jun B proto-oncogene</td>
</tr>
<tr>
<td>Kcn1a:</td>
<td>potassium voltage-gated channel, shaker-related subfamily, member 1</td>
</tr>
<tr>
<td>Kcnf1:</td>
<td>potassium voltage-gated channel, subfamily F, member 1</td>
</tr>
<tr>
<td>Kcnk12:</td>
<td>potassium channel, subfamily K, member 12</td>
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<tr>
<td>Klf2:</td>
<td>Kruppel-like factor 2 (lung) (predicted)</td>
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<td>Klf4:</td>
<td>Kruppel-like factor 4</td>
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<td>Klf10:</td>
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</tr>
<tr>
<td>Lcn7:</td>
<td>lipocalin 7</td>
</tr>
<tr>
<td>Lfng:</td>
<td>LFNG O-fucosylpeptide 3-beta-N-acetylglucosaminyltransferase</td>
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<td>Lims2:</td>
<td>LIM and senescent cell antigen like domains 2</td>
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<td>lipin 2 (predicted)</td>
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<td>Lrg1:</td>
<td>leucine-rich alpha-2-glycoprotein 1</td>
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<td>Madh3:</td>
<td>MAD homolog 3</td>
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<tr>
<td>Map2k3:</td>
<td>mitogen activated protein kinase kinase 3</td>
</tr>
<tr>
<td>Map3k6:</td>
<td>mitogen-activated protein kinase kinase kinase 6 (predicted)</td>
</tr>
</tbody>
</table>
Max: Max protein
Medl19: mediator of RNA polymerase II transcription, subunit 19 homolog
MEK: MAP kinase-ERK kinase
Mertk: c-mer proto-oncogene tyrosine kinase
Mgl1: macrophage galactose N-acetyl-galactosamine specific lectin 1
Midn: midnolin (predicted)
Mkl1: megakaryoblastic leukemia (translocation) 1
Mrc1: mannose receptor, C type 1
Mybbp1a: MYB binding protein (P160) 1a
Mycn: v-myc myelocytomatosis viral related oncogene, neuroblastoma derived
Nab2: Ngfi-A binding protein 2
Ndrg1: N-myc downstream regulated gene 1
Nedd4l: neural precursor cell expressed, developmentally down-regulated 4-like
Nfkbia: nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
Nid67: putative small membrane protein NID67
Nr4a1: nuclear receptor subfamily 4, group A, member 1
Nr4a3: nuclear receptor subfamily 4, group A, member 3
Nppc: natriuretic peptide precursor type C
PACAP: pituitary adenylate cyclase-activating polypeptide
Pal2g3: phospholipase A2, group III (predicted)
Pde10a: phosphodiesterase 10A
Pdk4: pyruvate dehydrogenase kinase, isozyme 4
Pdlm7: PDZ and LIM domain protein 7
Per1: period homolog 1
Per2: period homolog 2
Pim3: serine/threonine-protein kinase pim-3
Pkp2: plakophilin 2
Plld1: phospholipase D1
Plekhi1: pleckstrin homology domain containing, family F (with FYVE domain) member 1
Pnp1a2: patatin-like phospholipase domain containing 2 (predicted)
Polr2c: polymerase (RNA) II (DNA directed) polypeptide C, 33kDa
Prim2: DNA primase, p58 subunit
Prss11: HtrA serine peptidase 1
Prr5: proline rich 5
Ptp51: protein tyrosine phosphatase, receptor type, U
Pwwp2: PWWP domain containing 2B
Pxn: paxillin
Ralgds: ral guanine nucleotide dissociation stimulator
Rasd1: RAS, dexamethasone-induced 1
Rasgflc: RasGEF domain family, member 1C (predicted)
Rasgrp3: RAS, guanyl releasing protein 3 (predicted)
Rassf5: ras association (RalGDS/AF-6) domain family 5
Rbm3: RNA binding motif (RNP1, RRM) protein 3
Ret: ret proto-oncogene
Rimbpl: RIMS binding protein 2
Rin3: Ras and Rab interactor 3
Rkhd3: ring finger and KH domain containing 3 (predicted)
Rnf39: ring finger protein 39
Runde1: RUN domain containing 1 (predicted)
Sbk: SH3-binding domain kinase 1
Sesn1: sestrin 1 (predicted)
Sgk: serum/glucocorticoid regulated kinase
Siat7E: sialyltransferase 7E
Slc21a14: solute carrier organic anion transporter family, member 1c1
Slc25a25: solute carrier family 25 (mitochondrial carrier, phosphate carrier), member 25
Slc2a1: solute carrier family 2 (facilitated glucose transporter), member 1
Slc39a8: solute carrier family 39 (zinc transporter), member 8
Slc40a1: solute carrier family 40 (iron-regulated transporter), member 1
Slc9a3r2: solute carrier family 9 (sodium/hydrogen exchanger), member 3 regulator 2
Slit2: slit homolog 2
Smpdl3b: sphingomyelin phosphodiesterase, acid-like 3B
Snflk: SNF1-like kinase
Spry4: sprouty homolog 4 (predicted)
Spsb1: splA/ryanodine receptor domain and SOCS box containing 1 (predicted)
Srxn1: sulfiredoxin 1 homolog
Sstr2: somatostatin receptor 2
Sta2: stefin A2 (predicted)
Sult1a1: sulfotransferase family, cytosolic, 1A, phenol-preferring, member 1
Tcfcp2l1: transcription factor CP2-like 1
Tcfcp2l2: transcription factor CP2-like 2
Timp3: tissue inhibitor of metalloproteinase 3
Tiparp: TCDD-inducible poly(ADP-ribose) polymerase (predicted)
Tmem10: transmembrane protein 10
Tnfrsf11b: tumor necrosis factor receptor superfamily, member 11b
Trib1: tribbles homolog 1
Tsc22d3: TSC22 domain family, member 3
Uae1: urinary albumin excretion QTL 1
Usp43: ubiquitin specific protease 43 (predicted)
Usp54: ubiquitin specific peptidase 54
Vdac1: voltage-dependent anion channel 1
Vgll4: vestigial like 4
Vipr1: vasoactive intestinal peptide receptor 1
Vwa1: von Willebrand factor A domain containing 1
Wnt-2: wingless-related MMTV integration site 2
Wrnip1: Werner helicase interacting protein 1
Xkr6: X Kell blood group precursor related family member 6 homolog
Xdh: xanthine dehydrogenase
Zcch8: zinc finger, CCHC domain containing 8 (predicted)
Zfp189: zinc finger protein 189 (predicted)
Effect of Pyrethroid Insecticides on Gene Expression in the Mammalian Central Nervous System

Introductory Chapter

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I. Overview

Pyrethroids are neurotoxicants that disrupt nervous system function by interacting with membrane bound ion channels in neuronal plasma membranes. Pyrethroid induced alterations in ion channel function result in changes in neuronal firing patterns. These altered firing patterns underlie the acute symptoms of pyrethroid intoxication observed in vivo. At the molecular level, changes in the frequency or pattern of neuronal firing results in transcriptional induction or repression of activity-regulated genes. Changes in the expression of activity-regulated genes are an immediate response of neurons to excitatory or inhibitory stimuli and may lead to adaptive changes in the form or function of mature and developing neurons. Transient changes in gene expression can result in longer-lasting alterations in neuronal function that persist even when the excitatory stimulus (such as pharmacological excitotoxicants, i.e. a pyrethroid) is eliminated from the target tissue. To date, there is a paucity of data concerning alterations in gene expression and adaptive changes in neuronal form or function that occur downstream of the pharmacological action of pyrethroids at the neuronal membrane.

The present research was designed to test the hypothesis that in vivo pyrethroid exposures alter activity-regulated gene expression in neurons of the central nervous system. There were two specific aims in this research project. Specific Aim 1 determined whether gene transcription in the rat cortex is affected by pyrethroids in vivo, at doses that do not produce profound symptoms of pyrethroid poisoning. The goals of this specific aim included: 1) identifying genes that are transcriptionally induced or repressed by acute pyrethroid exposure, 2) characterization of the time course of gene expression following acute exposures, and 3) comparison of the similarities and differences in dose-
dependent gene expression patterns between a model Type I and Type II pyrethroid. A corollary hypothesis that pyrethroid exposures result in alterations in neuronal morphogenesis was also tested in light of the findings from the first phase of experimentation. Furthermore, a gene candidate believed to be driven by the pharmacological actions of pyrethroids at the neuronal membrane and linked to pyrethroid effects on neurite branching morphogenesis was further examined in the second chapter of this work. Specific Aim 2 tested whether acute exposure to equipotent doses of multiple Type I and Type II pyrethroids in vivo resulted in similar alterations in gene expression patterns: i.e., is there a structure-activity relationship for the transcriptional effects of pyrethroids? The goals under this specific aim included: 1) identification of suites of up- and down-regulated genes for each of six pyrethroids tested in an acute time course model, 2) cross compound enrichment analysis and qRT-PCR analysis of genes altered by the individual pyrethroids, and 3) exploration of gene networks commonly affected by the pyrethroid test panel.

The present data contribute to the development of a comprehensive mode-of-action model for the pyrethroids and provide insight into an aspect of pyrethroid neurotoxicology that to date remain poorly characterized.

II. Pyrethroid usage

Pyrethroids are a class of insecticides used in both agricultural and domestic applications including crop protection, household pest control, public health (i.e. head louse treatment), protection of textiles and in the control of contagious disease vectors, such as mosquitoes (Heudorf & Angerer 2001; Yanez et al. 2002). Usage of pyrethroids has increased in recent years due to the phase out of other insecticides such as organophosphates
and organochlorines (Amweg et al. 2005). Pyrethroids are now offered in a variety of commercial formulations available to ordinary consumers for use in the home. It is now estimated that approximately 80-90% percent of households in the United States use pesticides with pyrethroids comprising a considerable percentage of total use (Whyatt et al. 2003).

Increasing usage of pyrethroids, both in agricultural and domestic sectors, yields an increased likelihood of human exposure. Pyrethroids and pyrethroid metabolites have been detected in the blood of pregnant urban mothers, the urine of pesticide applicators, agricultural soil run-off, residential carpet dust, and, perhaps most importantly, in wipe samples from child-care centers (Leng et al. 1997; Whyatt et al. 2003; Colt et al. 2004; Weston et al. 2004; Tulve et al. 2006). The Food Quality Protection Act of 1996 mandates that the United States Environmental Protection Agency reexamine the established tolerances for various pesticide usages and, where necessary, consider the cumulative risk posed by exposure to multiple pesticides with same mechanism-of-action. As result, a variety of research efforts have been focused on examining the molecular mechanisms of pyrethroid toxicity and addressing data gaps critical to the establishment of a comprehensive mode-of-action model for these compounds (Soderlund et al. 2002).

III. Pyrethroid toxicity

Pyrethroids are neurotoxicants that pharmacologically alter the function of voltage-sensitive (and perhaps ligand-gated) ion channels located in the plasma membrane of neurons (Narahashi et al. 1996; Ray 2001; Soderlund et al. 2002). Figure 1.1 outlines the hypothesized mode-of-action for the acute effects of pyrethroids. Classically, pyrethroids have been divided into two types, Type I and Type II, based on chemical structure (Figure
1.2) and the acute poisoning syndromes observed in non-target species at near-lethal dose levels (Vijverberg and Van den Bercken 1990; Soderlund et al. 2002). Structurally, pyrethroids are esters of cyclopropane carboxylic acids linked to aromatic alcohols through a central ester bond (Kaneko and Miyamoto 2001). The difference in chemical structure between the two pyrethroid types is that Type II pyrethroids have an α-cyano group added to the alcohol moiety (Figure 1.2, arrow); Type I pyrethroids lack this group (Coats 1990). At high doses, the signs of acute Type I pyrethroid poisoning in the rat progress from episodes of aggressive sparring behavior and increased sensitivity to external stimuli to fine tremors followed by whole body tremors and prostration. This is known at the T-syndrome of pyrethroid poisoning. Type II pyrethroids produce a CS-syndrome characterized by increased pawing and burrowing behavior followed by profuse salivation, course whole body tremor and a ‘sinuous writhing’ motion known as choreoathetosis (Verschoyle and Aldridge 1980; Lawrence and Casida 1982). Acute disruption of neuronal firing within the nervous system is the underlying cause of both the Type I and Type II poisoning syndromes (Soderlund et al. 2002; Ray and Fry 2006). However, at this time the precise molecular mechanisms that underlie the divergent signs of poisoning across the two pyrethroid types are unknown.

Pharmacokinetic studies indicate that pyrethroids selectively partition into nervous system tissue from the blood due to their high lipophilicity (Ladowski 2002; Mirfazaelian et al. 2006). Accumulation of target tissue concentrations following oral exposure is rapid: i.e. brain concentrations of deltamethrin peak ~3 hr and elimination half-lives of pyrethroids range from approximately 10 – 30 hr (Ruzo et al. 1978; Anadon et al. 1991; Anadon et al. 1996; Anadon et al. 2006; Mirfazaelian et al. 2006). Metabolism of pyrethroids occurs via
oxidative cleavage of the central ester bond followed by hydroxylation of aromatic moieties (or vice versa) followed by phase II conjugation of hydroxylated products and elimination from the body (Soderlund et al. 2002). Cleavage of the central ester bond is the critical event in the deactivation of pyrethroids. Onset of the acute symptoms of pyrethroid poisoning correlate well with the accumulation of these compounds in nervous system tissue, and recover upon elimination of pyrethroid from the target tissues (Gray et al. 1980; Gray and Rickard 1982; Rickard and Brodie 1985; Kim et al. 2007).

There is no concrete evidence that pyrethroids produce significant toxicities apart from their actions in the nervous system. Some pyrethroids have been shown to have genotoxic effects in in vitro carcinogenesis assays (Villarini et al. 1998; Naravaneni and Jamil 2005; Undeger and Basaran 2005; Patel et al. 2006). However several two-year carcinogenicity studies in rats and mice provide little evidence of increased cancer incidence following chronic pyrethroid exposure (Parker et al. 1983; Cabral et al. 1990; Cabral and Galendo 1990). The International Agency for Research in Cancer (IARC) has concluded that there is insufficient evidence to classify pyrethroids as carcinogens (IARC 1991a, 1991b, 1991c). Nerve degeneration or the development of gross lesions either in the central or peripheral nervous system was not reported in studies of chronic pyrethroid exposures, however pyrethroid effects on neuronal microstructure (i.e. dendritic branching, number of synaptic contacts, myelination, etc.,) have not been examined.

Early research in the 1970’s that characterized the T- and CS- syndromes was all conducted at extremely high (i.e. lethal) dose-levels. At administered doses below those that induce frank signs of poisoning, pyrethroids affect a number of neurobehavioral endpoints. Reviews of these behavioral effects is available in Soderlund et al. (2002) and Wolansky and
Harrill (2008). Much like the acute signs of pyrethroid poisoning, induction of these acute behavioral effects is rapid, occurring within minutes to hours following oral exposure. Currently, motor activity is the most extensively characterized behavioral endpoint affected by pyrethroids. All pyrethroids, regardless of type, produce a decrease in ambulatory motor activity (Wolansky et al. 2006). Motor activity is an apical behavior shown to be a reliable and sensitive endpoint for measuring the neurotoxicological effects of pyrethroids (Crofton and Reiter 1984; Crofton and Reiter 1988; Crofton et al. 1991; Wolansky et al. 2006; Wolansky and Harrill, 2008). Administered doses required to reach the threshold for effects on motor activity range from ~0.5 to ~120 mg/kg (depending upon the compound) and are 1.5 to 2 orders of magnitude less than reported LD$_{50}$’s (Wolansky et al. 2006). Effects on motor function (and other behavioral endpoints) following acute pyrethroid exposures are transient. Full recovery to control performance levels is observed in a matter of hours following an acute dose (Crofton and Reiter 1984; Peele and Crofton 1987; McDaniel and Moser, 1993).

There is a paucity of data regarding repeated low-level pyrethroid exposures on neurobehavioral endpoints. The available data argue both for and against the development of deficits in nervous system function and cognition. A thirty day repeated exposure to 6 mg/kg cismethrin and 2 mg/kg deltamethrin did not detect any cumulative effects on ambulatory motor activity in adult rats (Crofton and Reiter 1984). Likewise, Hornyhova et al. (1995) did not detect any cumulative effects of supermethrin on learning and memory. On the other hand, Glowa (1986) demonstrated that a repeated exposure to 3 mg/kg deltamethrin (once a day for ten days) reduces daily pre-exposure response rates in an operant responding task and that response rates fail to return to controls levels after cessation of repeated dosing.
Additional studies are needed to clarify the effects of repeated pyrethroid exposures on nervous system function and cognition.

The actions of pyrethroids at the nerve membrane likely produce biological responses secondary to the neurotoxic effects observed in acute behavioral tests, one of which likely being changes in gene expression. In neurons, depolarizing excitatory stimuli activate intracellular signaling pathways that can trigger waves of *de novo* gene expression in the nucleus which can in turn lead to changes in the form or function of the cell (Bradley and Finkbeiner 2002; Clayton 2002; Adams and Dudek 2005). Transcription-mediated changes in neuronal function in response to excitatory stimulus may be beneficial, such as the induction of late-phase long term potentiation, a molecular correlate of learning and memory (Adams and Dudek 2005). In contrast, hyperexcitation of neuronal circuitry by a xenobiotic can lead to persistent detrimental effects on cell function as a result of *de novo* gene expression, such as during addiction to and withdrawal from drugs of abuse (Mohn et al. 2004; Nestler 2005). The acute behavioral effects of pyrethroids are driven by hyperexcitation of neuronal networks in the CNS. However, very few studies have examined the response of neurons to acute or repeated low-level pyrethroid exposure in terms of altered gene expression and changes in downstream cellular functions. The present experiments are designed to provide information on transcriptional events that occur in the brain in response to the acute pharmacological actions of pyrethroids at neuronal membranes *in vivo*. An additional benefit of this work is insight into pyrethroid-induced gene expression changes that may, in turn, lead to changes in neuronal function. An overview of the molecular mechanisms that control membrane excitability and gene expression in the adult nervous system is given below. In addition, a detailed description of the molecular and cellular
effects of pyrethroids and hypotheses on how the pharmacological actions of pyrethroids may affect adaptive gene expression in neurons is provided.

IV. Ion channel functions in the nervous system.

IVa. Molecular mechanisms of neuronal firing and synaptic communication.

In neurons, the rapid movements of Na\(^+\), K\(^+\) and Ca\(^{2+}\) ions across the plasma membrane are used to detect, integrate and transduce excitatory and inhibitory stimuli from post-synaptic regions of the dendritic arbor and cell soma to presynaptic axon terminals (McCormick 2003; Sheperd 2003a). Differential concentrations of ions on opposite sides of the plasma membrane establish a membrane potential, which is the molecular basis of electrical excitability in neurons (McCormick 2003). The propagation of changes in membrane potential (i.e. electrical activity) from the area of incoming dendritic synapses to outgoing axonal terminals is known as the action potential and is a key mechanism for intercellular communication in the nervous system (Sheperd 2003b). Initiation of the action potential is controlled by the selective conductance of ions through post-synaptic ionotropic neurotransmitter receptors in the dendrite or cell soma. Ion permeability through ionotropic receptors is increased by binding of their cognate neurotransmitters that are released from the pre-synaptic terminal of a neighboring neuron in response to an excitatory stimulus. The result is either an excitatory (EPSP) or inhibitory (IPSP) post-synaptic membrane potential intracellularly localized to the activated receptor complex (Byrne 2003). Two of the predominant ionotropic receptors present in neuronal networks of the cortex are excitatory glutamate receptors (both NMDA- and AMPA- subtypes) and inhibitory \(\gamma\)-amino butyric acid (GABA\(_A\)) receptor, which produce EPSPs and IPSPs, respectively (Waxham 2003). In the
dendritic tree of a single neuron, EPSPs and IPSPs are both temporally and spatially integrated to produce a change in the neuronal membrane potential (Sheperd 2003b).

Changes in membrane potential in the positive direction that reach the threshold for action potential firing result in the opening of axonal voltage-sensitive sodium channels (VSSCs), thereby allowing Na\(^+\) to rapidly flow through the neuronal membrane and depolarize the cell. As the cell becomes more depolarized, VSSCs deactivate (terminating Na\(^+\) conductance into the cell) and voltage-sensitive K\(^+\) channels (“delayed rectifier channels”) open. K\(^+\) flows out of the cell to offset the net effect of Na\(^+\) ion movement on the membrane potential. The action potential is propagated down the axon by clusters of voltage-sensitive Na\(^+\) and K\(^+\) channels sub-cellularly localized to patches of unmyelinated neuronal membrane (McCormick 2003). Voltage-sensitive Ca\(^{+2}\) channels (VSSCs) found in the dendrites, soma and axon terminals of neurons also contribute to changes in membrane potential with different sub-types (L-, N- and P/Q) mediating divergent intracellular events secondary to the action potential (see next section). When the action potential reaches the axon terminals, neurotransmitter filled intracellular vesicles fuse with the plasma membrane. This process is dependent upon the entry of Ca\(^{+2}\) into the cell cytoplasm via N-Type VSCCs (Schwarz 2003). Neurotransmitters then diffuse across the synaptic cleft, interact with post-synaptic receptors in neighboring cells and elicit responses such as increases in excitatory (glutamate) or inhibitory (GABA) tone in post-synaptic neurons or movement in innervated muscle cells (Deutch and Roth 2003).

The rapid, transient increases in ionic conductance that control the membrane potential and trigger neurotransmitter release are the molecular basis for intercellular communication in the CNS, both between neighboring neurons and between neurons and
innervated tissues (McCormick 2003). There is ample experimental evidence that pyrethroid insecticides interfere with the function of at least one key molecular mediator of this process, namely VSSCs. There is limited evidence that Ca\textsuperscript{2+} and Cl\textsuperscript{−} channels that contribute to the maintenance of the membrane potential are sensitive to pyrethroids (Narahashi et al. 1996; Soderlund et al. 2002; Ray et al. 2006). There is also evidence that pyrethroids may interfere with the mechanisms of neurotransmitter release independent of their actions on action potential firing. All these effects likely contribute to the neurotoxic actions of pyrethroids.

**IVb. Influence of neuronal activity on gene expression.**

Electrical activity at the neuronal plasma membrane is not only a mechanism of rapid intercellular communication in the nervous system but also serves as the initiating event in a number of intracellular signaling cascades that control both transient and persistent responses of the neuron to excitatory and inhibitory stimuli (Finkbeiner and Greenberg 1998; Zhang and Poo 2001; Wong and Ghosh 2002). A critical event mediating these adaptive responses is *de novo* gene expression. Gene expression leads to increased translation of effector proteins which can then modulate cell function (Clayton 2000). Gene transcription in the cell nucleus is coupled to changes in the function of membrane bound ion channels and neurotransmitter receptors through increases in the intracellular concentrations of second messenger molecules. These second messengers activate or inhibit signaling transduction pathways that terminate in the cell nucleus and affect the activity of constitutively-expressed transcription factors (Schulman and Roberts 2003; Fields et al 2005). A critical second messenger that activates such signal transduction pathways is Ca\textsuperscript{42+} (Schulman and Roberts 2003). Increases in intracellular Ca\textsuperscript{2+} concentrations ([Ca\textsuperscript{2+}]\textsubscript{i}) can occur through a variety of mechanisms in response to changes in neuronal excitation. These include opening of
voltage-sensitive Ca\(^{2+}\) channels (VSCC) during depolarization, activation of NMDA and AMPA-type glutamate receptors in response to pre-synaptic neurotransmitter release and activation of metabotropic glutamate receptors that increase intracellular inositol-1,4,5-triphosphate (IP\(_3\)) and trigger Ca\(^{2+}\) release from intracellular stores (Larea et al. 1997; Finkbeiner and Greenberg 1998; Verkratsky 2002; Wang et al. 2004). Movement of Na\(^+\), K\(^+\) and Cl\(^-\) ions across the plasma membrane regulate changes in the membrane potential which controls activation of VSCCs, the release of neurotransmitters and subsequent activation of second messenger systems. Thus, even though these ions do not act directly to activate signaling cascades in the neuronal cytoplasm, they do influence the concentration of intracellular second messengers through electrical coupling of ion channels and neurotransmitter release systems.

Activity-dependent neuronal gene expression occurs regularly under normal physiological conditions in response to stimuli from the surrounding environment (Cancedda et al. 2003; Majdan and Shatz 2006; Tropea et al. 2006). However, changes in the frequency, amplitude and type of neuronal stimulation differentially affect the activation pattern of intracellular second messenger systems and may result in unique patterns of gene expression. For example, the frequency and not the duration, of increases in [Ca\(^{2+}\)]\(_i\) controls the expression of the immediate-early transcription factor \(c-fos\) (Fields et al. 1997), some neural cell adhesion molecules (Itoh et al. 1997) and a variety of genes related to synaptic transmission, cell growth and motility (Xiang et al. 2007).

Given that alterations in neuronal firing patterns mediate the transcriptional expression of a variety of downstream effectors, it is reasonable to assume that pharmacological agents or toxicants that alter neuronal firing patterns and the excitability of
neuronal networks may, in turn, trigger alterations in gene expression. This abnormal wave of gene expression could then result in a detrimental alteration in cellular microstructure or function. As detailed below, pyrethroids interact with one (and perhaps more than one) type of membrane-bound ion channel that regulates neuronal firing patterns and excitability. Therefore, the pharmacological actions of pyrethroids may be capable of inducing alterations in cell function via the abnormal induction of activity-dependent genes. The transcriptional response of nervous system tissue, downstream of the pharmacological interactions of pyrethroids at their molecular target sites, to date, has not been thoroughly explored.

V. Molecular targets of pyrethroids.

Va. Voltage-sensitive Na\(^+\) channels and pyrethroid effects on neuronal firing rates.

Voltage sensitive Na\(^+\) channels (VSSCs) are considered to be the principal molecular target of pyrethroids in both target and non-target species and are the most extensively characterized sites of pyrethroid action (Narahashi 2000; Soderlund and Knipple 2003). As noted above, VSSCs open in response to changes in the neuronal membrane potential toward more positive values and deactivate as the neuron becomes depolarized. Pyrethroids affect VSSC gating kinetics by slowing inactivation. The result is increased conductance of Na\(^+\) through VSSCs during membrane depolarization, a time when unmodified VSSCs are normally impermeable to Na\(^+\) (Narahashi 2000). This period of abnormal Na\(^+\) conductance is known as a Na\(^+\) tail current. The effect of pyrethroids on Na\(^+\) currents was first examined in invertebrate and amphibian nerve preparations (Lund and Narahashi 1981; Vijverberg et al. 1982). Further studies using heterologous expression systems (Choi and Soderlund 2006), NE-115 neuroblastoma cells (Chinn and Narahashi 1986) and primary cultures of rat dorsal root ganglia (Tatebayashi and Narahashi 1994), cerebellar Purkinje cells (Song and
Narahashi 1996) and hippocampal neurons (Motomura and Narahashi 2001) confirmed that pyrethroids induce prolonged Na\(^+\) tail currents in mammalian VSSCs. In the mammalian CNS, seven VSSC isoforms (\(\alpha\)-subunits) are expressed in complex overlapping patterns both with each other and with a variety of accessory proteins (\(\beta\)-subunits, Ogata and Ohishi 2002; Catterall 2003). The sensitivity of different VSSC \(\alpha\)-subunits to different pyrethroids may vary and the presence of \(\beta\)-subunits has been demonstrated to modify pyrethroid effects on Na\(^+\) conductance through the \(\alpha\)-subunit channel protein (Smith & Soderlund 1998; Soderlund et al. 2002; Choi & Soderlund 2006). A comprehensive characterization of the effects of different pyrethroids on VSSCs in the brain is not available. However, the available data from different labs using a variety of cellular preparations indicate that the qualitative characteristics of VSSC modification are similar for all pyrethroids: i.e. persistent inward Na\(^+\) tail currents occur during the period of normal channel deactivation. The duration of Na\(^+\) tail currents (defined as a time constant of decay, \(\tau\)) are variable for different compounds within the pyrethroid class, sometimes dramatically so. Typically, VSSC modification by Type I pyrethroids results in a Na\(^+\) tail current that lasts on the order of milliseconds while Type II pyrethroids produce Na\(^+\) tail currents that last on the order hundreds of milliseconds (Vijverberg and Van den Bercken 1990; Choi and Soderlund 2006).

The persistent Na\(^+\) tail currents produced by pyrethroids cause the membrane potential to be slightly more positive (i.e. closer to the threshold potential for neuronal firing) than what would be observed under normal physiological conditions. A consequence of this perturbation is an increase in the frequency of neuronal depolarization in the form of repetitive firing of action potentials following an excitatory stimulus. Multiple Type I and Type II pyrethroids have been shown to induce repetitive action potentials following a single
stimulus in the *X. laevis* peripheral nervous system (Vijverberg 1982) and invertebrate giant axons (Lund and Narahashi 1981, Lund and Narahashi 1982). In mammals, repetitive firing of spinal and trigeminal reflex arcs as well as caudate nuclei of the brain has been observed in response to an excitatory stimulus (Ray 1980; Forshaw and Ray 1986). In addition, neurons from a variety of brain regions have been shown to be sensitive to pyrethroids (Tatebayashi and Narahashi 1994; Song and Narahashi 1996a; 1996b; Motomura and Narahashi 2001; Ginsburg and Narahashi 2005; Meyer et al. 2007; Shafer et al. 2008). It is likely that the acute manifestations of pyrethroid toxicity are the result of the summated effect of disrupted firing patterns at multiple neural substrates throughout the CNS. Hyperexcitation of these brain regions could also conceivably result in the induction of activity-regulated genes that affect downstream neuronal functions.

There is also evidence that the rate of spontaneous neuronal firing (action potentials that occur in the absence of stimulus) is also affected by pyrethroids. Increased rates of spontaneous action potential firing have been observed in *X. laevis* lateral-line sense organs (Vijverberg and Van den Bercken 1990). An increase in spontaneous firing is also observed in unstimulated hippocampal and cortical networks, *in vitro* (Meyer et al. 2007, Shafer – personal communication). In contrast, *in vitro* studies by Meyer et al. (2007) and Shafer et al. (2008) observe decreases in the spontaneous firing rate of glutaminergic neuronal networks derived from the rat hippocampus, cerebrocortex and spinal cord when the influence of inhibitory GABAergic inputs is removed.

The development of persistent Na$^+$ tail currents and subsequent changes in neuronal firing patterns is the hypothesized molecular mechanism-of-action for acute pyrethroid toxicity (Figure 1.1). As detailed above, neurons respond to increases in neuronal excitation
through the induction of de novo gene expression. These gene expression changes in the adult brain are meant to augment the function of specifically activated neuronal circuits in response to a sensory stimulus (Watanabe et al. 1995; Singh et al. 1997; Hendry et al. 2003; Harwell et al. 2005). Acute exposure to pyrethroids could result in non-specific, global hyperexcitability of neuronal circuits within the brain and result in the abnormal over-expression of activity-dependent adaptive gene transcripts.

While the role of changes in spontaneous firing patterns in the development of acute pyrethroid poisoning symptoms in adult animals is unknown, it is highly likely that changes in the spontaneous activity of neurons in the developing nervous system would lead to detrimental alterations in interneuronal connectivity and cell function. Early in development, neurons rely on spontaneous electrical activity to mediate the outgrowth of neuronal processes and the formation of functional synapses (Wong and Ghosh 2002). Pyrethroid-induced disruption of spontaneous activity would likely have a detrimental effect on these developmental events. It is difficult to predict the nature of these effects given the contradictory reports on spontaneous activity in developed neurons and an incomplete knowledge of how pyrethroids interact with developing neurons.

**Vb. Pyrethroid effects on voltage-sensitive Ca\(^{+2}\) channels.**

Pyrethroids have also been shown to interact with a number of voltage-sensitive Ca\(^{+2}\) channel (VSCC) isoforms expressed in the nervous system, however pyrethroid mediated effects on these channels are not as well characterized as effects on VSSCs and remain controversial (Shafer and Meyer 2004). VSCC effects are observed in vitro at pyrethroid concentrations similar to those that produce effects on Na\(^+\) channel function (Symington et al. 2007a; 2007b). Much like VSSCs, multiple isoforms of VSCCs are expressed in the
mammalian nervous system. Different VSCC isoforms mediate different neuronal functions. Ca\textsubscript{v}1.3 (L-Type) channels present on neuronal cell bodies and in dendrites mediate the neuronal membrane potential and are linked to downstream intracellular signaling cascades that mediate activity-regulated gene transcription. Ca\textsubscript{v}2.1 (P/Q-Type) and Ca\textsubscript{v}2.2 (N-type) mediate neurotransmitter release of axon terminals and may also influence gene transcription. The function of Ca\textsubscript{v}3.1-Ca\textsubscript{v}3.3 (T-Type) channels is less well known but is thought to mediate oscillatory bursts in pace-maker cells of the thalamus (Catterall et al. 2005). The pharmacological effects of only a small number of pyrethroids on VSCCs have been reported. Hildebrand et al. (2004) demonstrated that allethrin (a Type I pyrethroid) inhibited Ca\textsuperscript{2+} currents through rat Ca\textsubscript{v}1.3 (L-Type), Ca\textsubscript{v}2.1 (P/Q-Type) and Ca\textsubscript{v}3.1 (T-Type) VSCCs expressed in human embryonic kidney cells. Hagiwara et al. (1988) also noted an inhibition of L- and T-Type VSCCs in cardiac node cells. More recent studies (Symington and Clark 2005; Symington and Clark 2007) have demonstrated that deltamethrin either inhibits or enhances Ca\textsuperscript{2+} influx through N-Type VSCCs depending on the phosphorylation state of the neuron. Shafer and Meyer (2004) have criticized this body of research and suggest a number of serious limitations, including: only \textit{in vitro} observations, lack of concentration-response relationships, the use of indirect measures of VSCC function, and contradictory results from different laboratories/preparations. The role of VSCCs in pyrethroid toxicity is clearly controversial at this time.

As neurotransmitter release is dependent upon entry of Ca\textsuperscript{2+} into the neuron, indirect evaluations of VSCC function can be made based on studies of pyrethroid-induced neurotransmitter release. Previous reports using a variety of experimental models consistently demonstrate that neurotransmitter release is increased in the presence of...
pyrethroids. Studies using synaptosomal preparations demonstrated increased neurotransmitter release in the presence of pyrethroids following a depolarizing stimulus (for review, see Shafer & Meyer 2004). These studies, however, did not examine the mechanisms of increased neurotransmitter release. More recent work (Symington and Clark 2005; Symington et al. 2007) also report increased Ca\(^{+2}\) entry into, and NT-release from, synaptosomes treated with deltamethrin. These effects on neurotransmitter release were abolished in the presence of \(\omega\)-conotoxin MVIIC, a Ca\(_{\text{v}}\)\(_{2.2}\) (N-type VSCC) channel blocker, indicating that this channel mediated the effect. However, in these same studies the Type I pyrethroid cismethrin also caused increased internal Ca\(^{+2}\) concentrations, with no effects on neurotransmitter release. The authors attribute this observation to differences in the route of Ca\(^{+2}\) entry into the synaptosomes enhanced by the two compounds. These observations suggest that the effects of different pyrethroids on Ca\(^{+2}\) entry may not be equivalent.

Pyrethroids have also been shown to have heterogeneous effects on neurotransmitter release \textit{in vivo}. A series of microdialysis studies sampled the extracellular milieu of various brain regions of conscious rats treated with a variety of pyrethroids and demonstrates that: 1) different pyrethroids produce non-homologous increases or decreases in neurotransmitter-release, 2) pyrethroid-mediated effects on neurotransmitter release differ depending on brain region and type of neurotransmitter, 3) dose-dependent, biphasic effects on neurotransmitter release may occur and 4) some high dose effects on neurotransmitter release are eliminated by treatment with VSCC antagonists independent of pyrethroid actions at sodium channels (Hossain et al. 2004; Hossain et al. 2006; Hossain et al. 2008). Finally, Meyer and Shafer (2006) have reported that permethrin (a Type I pyrethroid) and not deltamethrin (a Type II pyrethroid) caused increases in neurotransmitter release independent of pyrethroid actions at
VSSCs in cultured hippocampal neurons. In total, these observations indicate that neurotransmitter release is sensitive to pyrethroids. However, the specific effects of different pyrethroids on VSCCs and neurotransmitter release are difficult to predict, may differ between compounds, and may result in divergent patterns of activity-dependent gene expression between pyrethroids following an acute exposure in vivo.

Vc. Pyrethroid effects on voltage-sensitive Cl⁻ channels.

The role of voltage-sensitive Cl⁻ channels in the toxicity of pyrethroids is not well understood. A decrease in the probability of opening of a Cl⁻ permeable, Ca²⁺-independent voltage-sensitive anion channel has been reported for several Type II pyrethroids in NE-115 neuroblastoma cells (Burrr and Ray 2004). Entry of Cl⁻ into neurons through a voltage-sensitive anion channel could antagonize the effect of prolonged Na⁺ entry (through VSSCs) on the membrane potential. Therefore, inhibition of Cl⁻ conductance by pyrethroids would enhance VSSC-mediated pyrethroid effects on membrane excitability. Type II pyrethroid actions at this channel occur in vitro at concentrations comparable to those that produce effects on VSSC function (Ray and Fry 2006) However, the molecular composition, physiological properties and in vivo expression patterns of the pyrethroid sensitive anion channel identified in NE-115 cells has not been fully characterized. Therefore, the relevance of pyrethroid effects at this molecular target in vivo is yet to be established. There are no known instances where Cl⁻ acts directly as a second messenger in the neuronal cytoplasm. It is unlikely that Cl⁻ entry into neurons directly activates signaling networks that control gene expression. However, Cl⁻ entry may antagonize activity-dependent gene expression by maintaining the membrane potential at values lower than those needed to induce entry of Ca²⁺ into the cell through voltage-sensitive Ca²⁺ channels. Antagonizing Cl⁻ entry, in turn,
could lead to increased activation Ca$^{2+}$-dependent signaling networks that control activity-dependent gene expression.

**Vd. Pyrethroid effects on GABAergic receptors.**

A possible role for a direct effect of pyrethroids on GABA$\text{A}$ receptors has been vigorously investigated over the past 30 years. Inhibitory GABAergic interneurons are expressed throughout the mammalian cerebrocortex (Hof et al. 2003). These interneurons have synaptic contacts on the dendrites and cell soma of excitatory glutaminergic pyramidal neurons in the cortex. The neurotransmitter $\gamma$-amino butyric acid (GABA) is released from presynaptic terminals of interneurons, diffuses across the synaptic cleft, binds to postsynaptic GABA$\text{A}$ receptors and triggers Cl$^-$ entry into the postsynaptic neuron. This leads to hyperpolarization of the postsynaptic neuron and an overall decrease in neuronal excitability.

Radioligand binding experiments have demonstrated that Type II pyrethroids displace [$^{35}$S]TBPS from the picrotoxin binding site of GABA$\text{A}$ receptors in vitro (Lawrence and Casida, 1983; Lawrence et al. 1985; Crofton et al. 1987; Lummis et al. 1987; Olsen et al. 1989). Type I pyrethroids do not display this activity (Crofton et al. 1987). Displacement of GABA$\text{A}$ receptor ligands by Type II pyrethroids occurs at concentrations much higher (> 3 orders of magnitude) than those required to produce effects on VSSC-mediated Na$^+$ currents and actual blockade of Cl$^-$ influx was observed at even higher concentrations (Lawrence et al. 1985; Abalis 1986; Ray and Fry 2006). In contrast, some studies did not detect any modification of GABA$\text{A}$-mediated chloride conductance in the presence of Type II pyrethroids (Ogata et al. 1988). An argument against GABA$\text{A}$ receptor antagonism as a mechanism of pyrethroid toxicity in vivo is demonstrated by the studies of Gilbert et al. (1989), Joy et al. (1990) and Joy and Albertson (1991). These studies did not detect any
pyrethroid-mediated modulation of GABA<sub>A</sub> function in a pair-pulsed stimulus paradigm at high doses, but in fact observed the opposite of the predicted effect (i.e., reduced inhibition of neuronal activity) for only some high doses of the pyrethroids tested. All of these data indicate that an alteration in GABA<sub>A</sub> receptor function by pyrethroids does not occur at tissue concentrations comparable to those that produce effects on VSSC function. However, they do not exclude the possibility that antagonism (or agonism) of GABA<sub>A</sub> receptor function occurs at high administered doses of pyrethroids and contributes the divergent poisoning symptoms produced by the two different pyrethroid classes (Crofton and Reiter 1987). Given these findings, it is equally unlikely that a direct pharmacological action of pyrethroids at the GABA<sub>A</sub> receptor complex would contribute to alterations in activity-dependent gene expression patterns in vivo at administered doses that do not cause profound signs of pyrethroid poisoning.

VI. Pyrethroid effects on gene expression.

As noted above, modulation of VSSC or VSCC function and neurotransmitter release by pyrethroids provides several mechanisms whereby these compounds may influence activity-regulated gene expression in a neuron. Based on the pharmacological actions of pyrethroids, it can be hypothesized that these compounds cause changes in activity-regulated gene expression by altering the intracellular concentration of second messengers (i.e. Ca<sup>2+</sup>) that initiate and mediate activity-regulated transcription in the nucleus. This may occur via three distinct mechanisms. First, pyrethroids may interact with a molecular target site, such as VSCCs, that directly controls the pattern and duration of transient intracellular Ca<sup>2+</sup> concentrations. Second, pyrethroids may indirectly increase the intracellular levels of Ca<sup>2+</sup> by enhancing Na<sup>+</sup> influx (or blocking Cl<sup>-</sup> influx), which depolarizes the membrane potential...
and triggers activation of Ca\textsuperscript{2+} channels and Ca\textsuperscript{2+} entry. Lastly, pyrethroids may increase neurotransmitter release by producing repetitive firing patterns in presynaptic neurons, thereby simulating or inhibiting the excitatory state of post-synaptic neurons and activating multiple intracellular second messenger systems via neurotransmitter receptor stimulation. Based on the available data, the concentrations required to pharmacologically alter the function of the VSSC, VSCC and Cl- channel types are very similar (Ray and Fry 2006). This means that all of the pharmacological actions may be occurring simultaneously in an integrated neuronal circuit affected by pyrethroids.

This multitude of pharmacological actions, combined with the inherent complexity of intracellular signaling pathways known to control activity-regulated gene expression and an incomplete knowledge of the genes that may be affected by alterations in neuronal activity, make predictions regarding the effects of pyrethroids on gene expression and intracellular signaling extremely difficult. An experimental approach that empirically identifies and characterizes the transcriptional response of the nervous system to pyrethroids \textit{in vivo} serves as a starting point for the systematic examination the complex intracellular events affected by the integrated pharmacological actions of pyrethroids at the neuronal membrane. The overall framework for this molecular approach is to define gene expression changes that occur in response to pyrethroids \textit{in vivo}, validate these changes in an \textit{in vitro} model of functioning neuronal networks, and use pharmacological tools and biochemical manipulations to determine which molecular actions of the pyrethroids are mediating the response. The experiments conducted in the present study are designed to address the first component of this frame-work: i.e. the identification of activity-regulated gene expression changes.
in vivo. An additional benefit of this work is insight into potential changes in neuronal form or function that may occur downstream of pyrethroid effects on neuronal activity.

Previous research provides limited evidence that acute pyrethroid exposure alters the expression of some genes in the central nervous system. Hassouna et al. (1996) observed increases in c-fos and JunB immunoreactivity in multiple brain regions of Sprague-Dawley rats several hours after a single i.p. treatment with either permethrin or cypermethrin. In addition, Imamura et al. (2006) reported increased expression of brain-derived neurotrophic factor (Bdnf) in the cortex of rats acutely exposed to deltamethrin. Bdnf, c-fos and JunB are all immediate early genes (IEGs) whose expression is known to correlate with excitatory activation of neuronal circuits (Herdegen and Leah 1998; Fukuchi et al. 2005). Together, these data provide evidence that one of the responses of nervous system tissue in vivo to pyrethroid exposure is an upregulation in the expression of genes controled by neuronal activity.

In addition, changes in the expression of neurotransmitter synthesis and transport machinery have been observed following pyrethroid exposure. Studies by Gillette et al. (2003) and Elwan et al. (2006) report an upregulation of the striatal dopamine transporter following repeated i.p. exposure to permethrin and deltamethrin. In the Elwan et al. (2006) study increased expression was coupled with increased transporter activity. Furthermore, Liu et al. (2006) report decreases in tyrosine hydroxylase, the penultimate enzyme in monoaminergic NT synthesis, following ten days of deltamethrin exposure. This change in expression was coupled with a decrease in dopamine content. The relationship of these gene expression changes to pyrethroid-induced effects on neuronal firing is unknown. However, these data demonstrate that gene expression changes in response to pyrethroids can lead to
persistent alterations in neuronal function. Given these data, gene expression changes
induced by pyrethroids should be included in a comprehensive mode-of-action model
describing the toxicity of these compounds. However, there is currently a lack of data
concerning this aspect of pyrethroid neurotoxicity.

VII. Hypothesis and goals.

The present research was designed to test the hypothesis that in vivo pyrethroid
exposures result in changes in activity-regulated gene expression in neurons of the
central nervous system. There were two specific aims in this research project. Specific
Aim 1 determined whether gene transcription in the rat cortex is affected by
pyrethroids in vivo, at doses that do not produce profound symptoms of pyrethroid
poisoning. The goals of this specific aim included: 1) identifying genes that are
transcriptionally induced or repressed by acute pyrethroid exposure, 2) characterization of the
time course of gene expression following acute exposures, and 3) comparison of the
similarities and differences in dose-dependent gene expression patterns between a
representative Type I and Type II pyrethroid.

A corollary hypothesis that pyrethroid exposures result in alterations in neuronal
morphogenesis was also tested in light of the findings from the first phase of
experimentation. Furthermore, a gene candidate believed to be driven by the
pharmacological actions of pyrethroids at the neuronal membrane and linked to pyrethroid
effects on neurite branching morphogenesis was further examined in the second chapter of
this work.

Specific Aim 2 tested whether acute exposure to equipotent doses of multiple
Type I and Type II pyrethroids in vivo resulted in similar alterations in gene expression
patterns: i.e., is there a structure-activity relationship for the transcriptional effects of pyrethroids? The goals under this specific aim included: 1) identification of suites of up- and down-regulated genes for each of six pyrethroids tested in an acute time course model, 2) cross compound enrichment analysis and qRT-PCR analysis of genes altered by the individual pyrethroids, and 3) exploration of gene networks commonly affected by the pyrethroid test panel.

The present data contribute to the development of a comprehensive mode-of-action model for the pyrethroids and provide insight into an aspect of pyrethroid neurotoxicology that to date remain poorly characterized.

VIII. Experimental design.

Throughout the duration of this work an acute in vivo dosing paradigm was used to examine pyrethroid effects on gene expression. The acute exposure model was selected for a variety of reasons. First, the most extensively characterized, quantitative measures of pyrethroid toxicity are behaviors that have been examined using an acute dosing paradigm (see section 1 above and Wolansky et al. 2006). The present studies use exactly the same dosing conditions (species, strain, sex, age, route, vehicle and delivery volume) as used in these evaluations of acute toxicity in order to provide a reliable dose-metric with which to compare equipotent doses of pyrethroids and neurotoxic effect thresholds. In addition, extensive pharmacokinetic models for the test compounds used in the first chapter of this work (i.e. deltamethrin and permethrin) have been developed based in acute oral dosing studies (Mirfazaelian et al. 2006; Tornero-Velez et al. 2007). Estimates of the time-to-peak tissue concentrations and elimination half-lives in the brain helped guide the selection of time points used in the in vivo portion of these studies. Estimates of brain concentrations from
these models were also used to compare *in vivo* results to *in vitro* functional assays. Toxicological and pharmacokinetic profiles for sub-chronic or repeated dosing schedules with pyrethroids are not as extensively characterized as the acute dosing paradigms. Therefore, comparisons of relative potency or estimations of tissue dosimetry could not be made with a great deal of confidence if a repeated dosing schedule was used.

In the second chapter of this work high-throughput oligonucleotide microarrays are used for the identification of pyrethroid-sensitive genes and comparison of the global transcriptional responses of the rat frontal cortex between two “model” pyrethroids, deltamethrin (Type II) and permethrin (Type I). The frontal cortex was selected as the tissue of interest as previous studies have demonstrated increases in activity-regulated gene transcripts and disruption of neuronal firing patterns *in vivo* in this region of the brain following acute pyrethroid exposure (Ray 1980; Hassouna et al. 1996; Wu and Liu 2003). Linear and isotonic regression models (Tusher et al. 2002; Hu et al. 2005) were used to identify dose-dependent gene expression changes and thresholds for effects. For a select number of transcripts, gene expression changes are confirmed by qRT-PCR. In addition, an *in vitro* growth assay is used to examine a potentially neurotoxic effect of pyrethroids on neuronal morphology that was identified during functional category level analysis of microarray data. These *in vitro* data demonstrate that exposure to pyrethroids can stimulate abnormal changes in neuronal morphology, and also indicates that pyrethroids may act as developmental neurotoxicants.

Chapter Three focused on characterizing transcriptional and translational upregulation of Ca$^{2+}$/calmodulin-dependent protein kinase 1-gamma (*Camk1g*) by pyrethroids. *Camk1g* was identified in Chapter Two as a reliable indicator of pyrethroid exposure. Previous
studies in the literature indicated that this protein can mediate neuronal branching and growth and is transcriptionally regulated by changes in neuronal excitability. Increased expression of \textit{Camk1g} was identified and confirmed in the initial dose-response microarray study. Subsequent qRT-PCR analysis demonstrates that the \textit{Ca}\textasciitilde{}^{2+}-sensitive splice variant of \textit{Camk1g} (i.e. \textit{Camk1g1}) is upregulated by pyrethroids whereas the \textit{Ca}\textasciitilde{}^{2+}-insensitive splice variant of \textit{Camk1g} (\textit{Camk1g2}) is not. These results support a role for \textit{Ca}\textasciitilde{}^{2+} in the acute toxicity of pyrethroids.

Chapter Four expands upon the results of Chapter Two by comparing global gene expression profiles from the cortex of rats exposed to equipotent doses of either permethrin, bifenthrin or tefluthrin (Type I pyrethroids) or deltamethrin, cypermethrin or cyfluthrin (Type II compounds). Equipotent doses were once again based on the results of Wolansky et al. (2006). The LIMMA (Linear Models for Microarray Data) analysis method was used to detect treatment related changes in gene expression for each of the individual compounds (Smyth 2005). Suites of altered transcripts for each individual compound were then tested for enrichment in the expression profiles of other pyrethroids in the test panel using the SAFE algorithm (Barry et al. 2005). SAFE was also used to examine enrichment of GO categories and KEGG pathways in each compound in the test panel. Finally Igenuity® pathway analysis was used to construct a gene regulatory network commonly affected by the Type II pyrethroids in the test panel. Chapter Five summarizes the major findings, implications, and future directions of this work.
**Figure 1.1. Mode-of-action for the acute effects of pyrethroids.** The sequence outlined on the left of the figure corresponds to the sequential events leading from pyrethroid exposure to acute toxicity. Following an administered dose of pyrethroids accumulation of a target tissue (i.e. brain) dose occurs and is controlled by absorption, distribution, metabolism and elimination rates intrinsic to the system. In the target tissue, pyrethroids interact with a variety of voltage-sensitive ion channels, primarily voltage-sensitive sodium channels, which impact the membrane potential. Under normal physiological conditions an excitatory stimulus results in the firing of a single action potential as a means of intracellular communication in the nervous system. In presence of pyrethroids, a repetitive firing of the action potential is produced which leads to acute adverse effects at the behavioral level. These adverse effects range from mild effects on neurobehavioral endpoints such as ambulatory motor activity at low doses to profound signs of poisoning at high doses. The right side of the figure lists data gaps in the comprehensive mode-of-action of pyrethroids. Changes in voltage-sensitive ion channel function and membrane excitability can lead to subsequent changes in gene expression and adaptive or adverse changes in cellular form or function. To date, these types of effects have not been extensively characterized for pyrethroids.
Figure 1.2. *Examples of pyrethroid chemical structures.* Chemical structures of the Type I pyrethroid permethrin (top) and the Type II pyrethroid deltamethrin (bottom). A primary structural difference between the two types is the presence or absence of an α-cyano group on the alcohol moiety of the compound (arrow). The two compounds are used in Chapters 2 and 3 of the present work.


Transcriptional Response of Rat Frontal Cortex following Acute In Vivo Exposure to the Pyrethroid Insecticides Permethrin and Deltamethrin

Chapter 2

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Abstract.

Pyrethroids are neurotoxic pesticides that interact with membrane bound ion channels in neurons and disrupt nerve function. The purpose of this study was to characterize and explore changes in gene expression that occur in the nervous system subsequent to the pharmacological actions of pyrethroids at the neuronal membrane. Rats were acutely exposed to either deltamethrin (0.3 – 3 mg/kg) or permethrin (1 – 100 mg/kg) followed by collection of cortical tissue at 6 hours. The doses used range from those that cause minimal signs of intoxication in a behavioral test to doses well below apparent no effect levels. Affymetrix GeneChips® were used to obtain global gene expression profiles and detect changes in gene expression. A group of dose-responsive transcripts were identified using penalized linear (SAM) and isotonic (PIR) regression methods. A sub-set of these genes were confirmed by qRT-PCR. A separate set of rats were used to characterize the time course of transcript expression. Both differences and similarities in the transcriptional response were observed when comparing permethrin and deltamethrin. Changes in Camk1g, Ddc, Gpd3, c-fos and Egr1 mRNA levels were consistently observed across multiple test cohorts for both compounds. In addition, Significance Analysis of Function and Expression (SAFE) identified significantly enriched gene categories common for both pyrethroids including some related to branching morphogenesis and intracellular Ca\textsuperscript{2+} signaling. In vitro exposure of primary cortical cell cultures to both deltamethrin and permethrin resulted in an increase in the number of neurite branch points without effects on total neurite length. The effects on neuronal branching and gene expression identified here may represent novel aspects of pyrethroid neurotoxicity.
**Introduction.**

Pyrethroid insecticides are structural analogs of pyrethrum, a natural constituent of extracts from flowers of *Chrysanthemum cinerariaefolium*. Pyrethroids now represent a significant percentage of the world insecticide market (Amweg et al. 2005). This usage results in an increased potential for human exposure. Pyrethroid residues have been detected in sediments from agricultural run-off (Qin et al. 2006), residential dust samples (Colt et al. 2004), child-care centers (Tulve et al. 2006) and pyrethroid metabolites have been detected in human urine (Leng et al. 1997). A current focus in the field of pyrethroid research is examining the diverse neurotoxic mechanisms-of-action proposed for these compounds and determining if compounds belonging to this chemical class act through the same or similar mechanisms-of-action to produce similar adverse health outcomes (Soderlund et al. 2002).

Pyrethroids are neurotoxicants that disrupt nervous system function by interacting with membrane bound ion channels and altering their normal gating kinetics (Narahashi 1996). The primary molecular targets of pyrethroids are neuronal voltage-sensitive sodium channels (VSSCs, Catterall 1992; Ogata and Ohishi 2002). Prolongation of whole-cell Na$^+$ currents has been observed in a variety of cultured nervous system tissues exposed to pyrethroids (Song et al. 1996; Motomura and Narahashi 2001; Spencer et al. 2001; Ginsburg and Narahashi 2005). Furthermore, *in vitro* studies utilizing heterologous expression systems have demonstrated that pyrethroids increase sodium current through some mammalian VSSC isoforms (Na$_{1.2}$, Na$_{1.4}$ & Na$_{1.8}$) although the complete complement of mammalian VSSCs have not been examined for pyrethroid sensitivity (Smith and Soderlund 1998; Choi and Soderlund 2005; Shafer et al. 2005). Pyrethroids may also alter the gating kinetics of both neuronal voltage-sensitive Ca$^{2+}$ (VGCCs, Shafer et al. 2005; Syminton and Clark 2005).
and voltage-sensitive Cl channels (Burr and Ray 2004) at nominal concentrations similar to those that cause effects on VSSCs (Ray and Fry 2006). Isoforms of all of the aforementioned molecular targets are expressed in the plasma membrane of mammalian neuronal cells.

Pyrethroids affect mammalian nervous system function by producing hyperexcitability in neurons and increasing neuronal firing rates (Ray 1980; Wright et al. 1988; Vijverberg and van den Bercken 1990; Ray 2001). The acute manifestations of neurotoxicity observed in mammals following pyrethroid exposure are driven by this increased neuronal hyperexcitability which is in turn a result of the pharmacological actions of pyrethroids at their molecular target sites, primarily VSSCs (Ray 2001). Under normal conditions, neuronal membrane depolarization results in the activation of intracellular Ca\(^{2+}\)-dependent signaling pathways that control the induction of gene expression (Fields et al. 2005). In some cases, these transcriptional responses lead to persistent adaptive changes in cellular functions (i.e. neuronal plasticity; Bading 2000; McClung and Nestler 2008). Some neuroactive chemicals that are known to alter neuronal firing patterns or disrupt neurotransmission can trigger the induction of unique groups of gene transcripts which may in turn impact neuronal function (Bahi and Dreyer 2005; Cai et al. 2006; Xiang et al. 2007). While alterations in neuronal excitability is considered a critical event in the toxiciological mechanism(s)-of-action for pyrethroids, to date, the impact of pyrethroid-induced neuronal hyperexcitability on intracellular signaling pathways and inducible gene-regulatory networks have not been extensively examined.

In the present study Affymetrix GeneChip® microarrays were used to characterize the global transcriptional response of mammalian nervous system tissue following an acute oral exposure to two model pyrethroids: permethrin and deltamethrin. The dose ranges used
in this study include doses that cause minimal neurotoxic signs to doses well below apparent ‘no effect’ levels in in vivo behavioral tests (Wolansky et al. 2006). Low doses were used to minimize any potential transcriptional changes which may be due solely to excessive systemic toxicity at high pyrethroid doses. The goals of this study were to: 1) identify significant dose-dependent alterations in gene transcription, 2) determine at what point significant alterations in gene transcription occur along the dose-effect curve for pyrethroids and 3) determine if any of the dose-responsive genes are components of biological processes that may be sensitive to pyrethroids based on the hypothesized neurotoxic mechanisms-of-action for these compounds. Penalized linear (Significant Analysis of Microarrays, SAM) and isotonic (PIR) regression models (Tusher et al. 2001; Hu et al. 2005) as well as Significant Analysis of Function and Expression (SAFE, Barry et al. 2005) were used, respectively, to: 1) identify dose-responsive alterations in gene transcription and 2) identify Gene Ontology categories (Harris et al. 2004) and canonical cellular signaling networks (Kanehisha et al. 2004) that may be altered by pyrethroids. Alterations in transcript expression detected using microarrays were confirmed by qRT-PCR for a subset of pyrethroid-responsive genes. In addition, the time course of expression was characterized for a limited number of dose-responsive transcripts by qRT-PCR. Data from the in vivo gene expression studies lead to an investigation of pyrethroid effects on neuronal morphogenesis. Both permethrin and deltamethrin increased the number of branch points in a primary cortical cell culture with no effects on total neurite length or cell viability.
Methods.

**Chemicals.** Permethrin (3-phenoxybenzyl (1$R$,S)-cis-trans-3-(2,2-dichlorovinyl)-2,2-dimethyl-cyclopropanecarboxylate, 92.0% purity, isomer composition: 40% cis, 60% trans, 1:1 ratio of 1$R$ & 1$S$) and deltamethrin ((S)-cyano-(3-phenoxyphenyl)methyl (1$R$)-cis-3-(2,2-dibromovinyl)-2,2-dimethylcyclopropane carboxylate, 98.9% purity, isomer composition: 100% 1$R$, 3$R$, α$S$) were generously donated by FMC Corporation (Philadelphia, PA) and Bayer Cropscience (Research Triangle Park, NC), respectively. Pyrethroids were dissolved in corn oil (Sigma-Aldrich, St. Louis, MO) at 1, 10, 40 & 100 mg/mL permethrin and 0.3, 1 & 3 mg/mL deltamethrin. Dosing volume was 1mL/kg.

**Animal Care and Treatment.** Male Long-Evans rats (49-62 days of age) were obtained from Charles River Laboratories (Wilmington, MA) and housed two per cage in standard polycarbonate hanging cages (45 cm X 24 cm X 20 cm) with heat sterilized pine shavings for bedding (Beta Chips, Northeastern Products, Inc., Warrensburg, NY). Animals were maintained on 12h:12h photoperiod (lighted hours: 06:00-18:00) and allowed a 5-7 day period of acclimation to the colony prior to dosing. Colony rooms were maintained at 22.0 ± 2.0°C with a relative humidity of 55 ± 20%. Food (Purina 5001 Rat Chow) and tap water were provided ad libitum. The facility was approved by the American Association for Accreditation of Laboratory Animal Care (AAALAC) and all experimental procedures were approved in advance by the by the US EPA, National Health and Environmental Effects Research Laboratory Animal Care and Use Committee.

Four cohorts of animals were used in this study (Table 2.1). Cohort 1 was used for preliminary data collection to demonstrate that the selected doses of the two compounds
would alter gene transcription. Cohort 2 replicated these findings and expanded group sizes. Cohorts 1 & 2 were combined for microarray data analyses. Dose-response Cohort 3 was examined exclusively by quantitative real-time RT-PCR (qRT-PCR). Individual dose-response cohorts were exposed on separate days. All dosing occurred between 06:30 and 07:00 hours and the last test subject was euthanized before 18:00 hours. Dosing and euthanasia times for individuals were counterbalanced across time of day. Cohort 4 was used in qRT-PCR time course studies. In the time course studies rats were treated via oral gavage with 3 mg/kg deltamethrin, 100 mg/kg permethrin or vehicle. Each time point contained pyrethroid-treated and time-matched vehicle controls. All test subjects were dosed and euthanized between 07:30 & 17:30 hours. In every experimental cohort, test subjects were removed from the colony suite one hour prior to dosing and allowed to acclimate in a quiet holding room maintained under similar environmental conditions. Subjects were administered a single oral dose of test compound by gavage (1 mL/kg delivery volume), and allowed to recover in their home cage prior to euthanasia at 6 hours (dose-response studies) or 1, 3, 6 or 9 hours (time course studies). Subjects were then individually removed to an adjoining suite with a separate HVAC system for euthanasia by decapitation.

Whole brains were rapidly removed and placed on a cold plate (4°C). The cerebral cortex was removed by making a vertical incision at the anterior edge of the optic tract with a stainless steel razor, and rapidly frozen on a bed of dry ice. Cortical samples, without striatal tissue, were then bisected into contralateral hemispheres, weighed, frozen in liquid nitrogen and stored at -80°C.
**RNA Extraction.** Cortical samples were homogenized in 1 mL of TRI Reagent (Molecular Research Center, Inc., Cincinnati, OH) per 50-100 mg of tissue using a Polytron® PT-K homogenizer (Kinematica, Lucerne, Switzerland) and total RNA was isolated per manufacturer’s instructions. Total RNA pellets suspended in DEPC-treated H₂O were then subject to DNase I treatment and re-extracted with acid:phenol chloroform, pH = 4.7 (Ambion Inc., Austin, TX) and chloroform according to manufacturer’s protocol and re-suspended in DEPC-treated H₂O until use. The total RNA concentration of each sample was determined (absorbance @ 260 nm) on a Beckman-Coulter DU® 800 spectrophotometer (Fullerton, CA) and adjusted to 1.0 µg/µL prior to sample storage at -80°C. The ratio of absorbance values at 260 nm and 280 nm (Ab 260/280) was used to assess purity of total RNA samples. All samples used in these studies had Ab 260/280 ratios > 1.6 (data not shown). Preliminary PCR experiments using primers for rat β-actin genomic DNA (outlined in Tully et al. 2006) demonstrated that the above protocol adequately prevents genomic DNA contamination of total RNA samples (data not shown). In addition, the RNA integrity of each sample was determined using an Agilent 2100 Bioanalyzer and RNA 6000 Nano LabChip Kit (Waldbron, Germany) according to manufacturer’s instructions. All samples used in microarray and qRT-PCR experiments had 18S:28S rRNA ratios > 1.6 (data not shown). Following the RNA purity and integrity screens, aliquots of each total RNA sample (1 µg/µL for microarray hybridization or 25 ng/µL for qRT-PCR assays) were stored at -80°C until use.

**Microarray design, sample preparation and data collection.** All protocols for microarray sample preparation (except total RNA extraction, as above), GeneChip® hybridization, array
scanning and data collection were performed by Expression Analysis, Inc., (Durham, NC). Affymetrix Rat Genome 230 2.0 GeneChip® oligonucleotide microarrays (Santa Clara, CA) were used in this experiment. These microarrays contain 31,042 probe sets that represent ~28,000 unique \textit{Rattus norvegicus} genes and expressed sequence tags (ESTs). First and second strand cDNA synthesis, RNase H digestion and (ds)cDNA isolation for each sample were performed according to manufacturer’s protocol (Affymetrix 2004). Synthesis and clean-up of biotin-labeled cRNA was performed using a BioArray\textsuperscript{TM} High Yield\textsuperscript{TM} RNA transcript labeling kit (Enzo Life Sciences, Farmingdale, NY) and Qiagen RNeasy spin columns (Spoorstraat, Netherlands), respectively, according to manufacturer’s instructions. Biotin-labeled cRNA was fragmented using Affymetrix 5X fragmentation buffer (200mM Tris acetate pH=8.1, 100 mM KOAc, 150 mM MgOAc).

Fragmentation of biotin-labeled cRNA, GeneChip® hybridizations, washes and staining were performed according to standard Affymetrix protocols (Affymetrix 2004). Hybridizations were performed in an Affymetrix Hybridization Oven 640. Washes were performed on an Affymetrix Fluidics Station 450 using the EukGE-WS2v4-450 fluidics script. GeneChips® were scanned using an Affymetrix GeneChip® 3000 Scanner with the GCOS v1.2 software package. Target intensity was set to a value of 500 with all other scanning parameters set at the factory defaults. The 3’/5’ ratios for GAPDH and β-actin internal controls genes ranged between 0.93 – 1.11 and 1.2 – 2.01, respectively, indicating that degradation of RNA did not occur during sample preparation for hybridization. The intensity of hybridization controls (\textit{BioB, BioC, BioD} and \textit{Cre}) increased linearly on all arrays. Gene expression profiles for this experiment have been archived in the NCBI Gene Expression Omnibus (GEO) repository with the series accession number GSE7955.
Microarray Data Analysis. Two methods for the calculation of probe set expression summaries were compared: GeneChip® Operating Software v1.2 signal algorithm (GCOS, Affymetrix 2002) and Robust Multiarray Average (RMA, Irizarry et al. 2003). RMA expression summaries were calculated using RMAExpress© v.4.7 (University of California at Berkeley). RMA provided less within group variation in expression summary values as compared to GCOS v1.2, especially with probe sets with low fluorescent intensities (see Appendix A, Table 1). This is consistent with previous comparisons of the two methods (Millenaar et al. 2006) RMA expression summaries were used for the remainder of microarray data analysis. Microarray data from Cohorts 1 & 2 were combined for statistical analysis providing sample sizes of twelve for the control group and eight for each dose condition.

Analysis of dose-response relationships were performed using Significance Analysis of Microarrays (SAM, Tusher et al. 2001, version 2.21), with the quantitative/linear regression modeling component (SAM User’s Manual, Chu et al. 2005). In addition to identifying dose-responsive genes, SAM provides permutation-based estimates of the false-discovery rate ($FDR$) associated with lists of genes for which the null hypothesis is rejected. The SAM statistic ($d_i$) penalizes lowly expressed genes, and is most powerful when the dose-response function is nearly linear in the range examined. To potentially increase power and account for non-linearity in dose-response relationships, the SAM analyses were supplemented by penalized isotonic regression (PIR) according to the method of (Hu et al. 2005) which was specifically designed for microarray analysis. Similar to SAM, PIR penalizes lowly expressed genes and provides a permutation-based estimate of the false
discovery rate. In contrast to SAM, PIR allows for the dose-response relationship to be nonlinear, but assumes the relationship is increasing or decreasing as a function of increasing dose, and not the reverse direction. This method results in a score (the \( M \)-statistic) for each probe set that quantifies the evidence for an increasing or decreasing dose-response relationship.

To ensure that the rigorously conservative, permutation-based approaches for controlling Type I error did not exclude probe sets with dose-dependent increases or decreases in expression, an additional analysis was conducted with each regression model. Empirical \( p \)-values from the PIR analysis or SAM analysis were used to filter out probe sets with no apparent dose-related changes in expression (threshold \( p \)-value < 0.01). The reduced group of probe sets were then analyzed using a one-way analysis of variance (ANOVA) followed by a Benjamini-Hochberg correction for control of multiple comparisons. Dose was used as the independent factor. Probe sets meeting the Benjamini-Hochberg correction at an \( FDR < 0.05 \) were included in the gene lists of interest for each compound (Tables 2.2 & 2.3), analysis of dose thresholds for transcriptional changes (Figure 2.2) and the comparison of effects between compounds (Figure 2.3). Genes of interest for qRT-PCR confirmation were ranked based on the results of the SAM and PIR analyses in conjunction with the results of the one-way ANOVA. For probe sets that passed the one-way ANOVA significance threshold, a Dunnett’s multiple-comparison mean contrast test (Dunnett, 1955) was performed comparing the means of the respectively dose groups to the mean of the control group. Regression analysis were performed using R© version 2.3.0 statistical computing analysis software. Dunnett’s tests were performed using SAS v8.1 (SAS Institute, Inc., Cary, NC).
**Quantitative real-time RT-PCR.** qRT-PCR for each transcript of interest was performed using TaqMan® One-Step RT-PCR Master Mix Reagent Kits and TaqMan® Gene Expression Assays on a ABI 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA). AmpliTaq Gold® DNA Polymerase / dNTP mix, Multiscribe™ reverse transcriptase / RNA inhibitor mix and TaqMan® Gene expression primer-probe mix specific for the transcript of interest were combined according to manufacturer’s specifications (Applied Biosystems 2005). The reaction mixture was then dispensed into the reaction plate (15 µL / well) and 125 ng of total RNA (5 µL) was added. Each sample was measured in triplicate for each transcript of interest and an internal reference gene. Reaction plates were maintained at 5°C during loading procedure. During data collection, reactions were incubated at 48°C for 45 min followed by incubation at 95°C for 10 min and 40 cycles of 94°C for 25 sec and 60°C for 1 min.

qRT-PCR assays were designed via the Applied Biosystems (ABI) primer / probe selection algorithm and bioinformatics pipeline (Applied Biosystems 2006). The amplification efficiency of each assay utilized in this experiment was examined using a serial dilution of pooled total RNA from rat frontal cortex. Efficiencies were calculated as: 

\[ E_x = 10^{(-1/m)} - 1 \]

where \( E \) is the amplification efficiency of target transcript \( x \) and \( m \) is the slope of threshold cycles vs log [total RNA concentration] across the range of dilutions (Applied Biosystems 2004). Assay identification numbers, context sequences, amplicon lengths and calculated amplification efficiencies are listed in Appendix A, Table 2.

Nine transcripts identified as being up- or down-regulated were examined by qRT-PCR to confirm the dose-responsive trends observed during microarray analysis (see arrows,
Tables 2.2 & 2.3). The time course of expression for a number of transcripts identified as
dose-responsive was also examined in Cohort 4. qRT-PCR data from deltamethrin and
permethrin dose-response and time course studies were analyzed according to the $2^{-\Delta \Delta C_T}$
method as described by (Livak and Schmittgen 2001). β-actin expression did not change as a
function of time or dose for either compound (data not shown) and was used at the internal
reference for all $2^{-\Delta \Delta C_T}$ calculations. For dose-response studies, the mean $\Delta \Delta C_T$ of vehicle
treated controls were used as the $2^{-\Delta \Delta C_T}$ calibrator (Livak and Schmittgen 2001) to obtain
approximations of fold-change from control. For time course studies, the mean $\Delta \Delta CT$ of
vehicle treated controls were used as the $2^{-\Delta \Delta C_T}$ calibrator for each time-matched treatment
group.

Data from Wolansky et al. (2006) were used to assign equipotent dose-levels (EDL)
to the administered doses used in the present study to provide a comparative dose-metric
between the two test compounds (see Table 2.1). Statistical analysis of qRT-PCR dose
response data was performed using a two-way ANOVA with compound and equipotent dose
level (EDL) as independent variables and $2^{-\Delta \Delta C_T}$ as the dependent variable followed by
Dunnett’s mean contrast test. Transcripts with a significant compound by EDL interaction
were further analyzed using a one-way ANOVA with dose as the independent variable
followed by Dunnett’s mean contrast test. Statistical analysis of time course data was
performed using a two-way ANOVA with time and treatment as independent variables and $2^{-\Delta \Delta C_T}$ as the dependent variable. Transcripts with a significant time*treatment interaction ($p < 0.05$) were additionally analyzed with a one-way ANOVA at each time point with treatment
as the independent variable ($p < 0.05$)
Significance Analysis of Function and Expression (SAFE). The SAFE method (Barry et al. 2005) was used to identify pathways/functional categories whose genes are coordinately regulated in a dose-dependent manner. SAFE is similar to other pathway enrichment procedures (e.g. DAVID, Dennis et al. 2003), but accounts for correlation in gene expression within pathways using array permutation to rigorously control error rates. SAFE and accompanying array annotation were loaded from Bioconductor (v.1.8, Gentleman et al. 2004), which includes 126 KEGG pathways, 700 GO-BP (Gene Ontology biological processes) pathways, 142 GO-CC (Gene Ontology cellular component) pathways and 307 GO-MF (Gene Ontology molecular functions) pathways. SAFE tests for enrichment of significant dose-response relationships for genes within each pathway, and accordingly we first calculated the linear regression dose-response $p$-value for each gene. Genes with a nominal $p$-value < 0.05 formed the gene list to which the enrichment analysis was performed. SAFE (SAFE manual, Barry 2006) enables the user to define a pathway enrichment statistic and a Pearson test of binomial proportions (Pearson 1911) was then implemented. The Pearson statistics is similar to Fisher’s exact test commonly employed in pathway enrichment testing (GSEA, Mootha et al. 2003), but does not consider the number of significant genes to have been fixed a priori (Barry et al. submitted). 10,000 permutations of dose levels were used by SAFE to assess the significance of the entire procedure, using the (Yekutieli and Benjamini 1999) procedure to control the $FDR$ while accounting for the multiple pathways/categories. All categories with an estimated $FDR < 0.1$ are reported in Table 2.4, Part I.
**Combining pathway evidence for the two pyrethroids.** One aim of using these statistical methods in this study was to identify gene categories showing enrichment for dose-responsiveness for both permethrin and deltamethrin. The Fisher combined $p$-value method (Fisher 1930) allows accrual of evidence across multiple hypotheses, and thus is ideal for testing combined evidence for enrichment of each pathway for both chemicals. Under the null hypothesis that neither chemical shows enrichment for the pathway, each of the two $p$-values is uniform $[0,1]$, and the Fisher statistic

$$S = -2(\ln(p_{\text{delta}})+\ln(p_{\text{norm}}))$$

is distributed as $\chi^2_2$. The Fisher approach has favorable optimality properties (Littell and Folks 1973) and results in a new (combined) $p$-value for each pathway. For the multiple pathways tested, the Benjamini-Hochberg (1995) method was applied to control the false discovery rate ($FDR < 0.1$).

Fisher’s statistic can be asymmetrically sensitive to very small $p$-values for a single chemical, even if the results for the other chemical are not significant. Thus, among pathways with a significant Fisher statistic, the focus was placed on those which showed SAFE $p$-values $< 0.05$ for both chemicals.

**Cell culture and treatment.** Cortical cultures containing neurons and glia were prepared from neocortices of newborn rat pups according to the protocol used by Chandler et al. (1993) with modifications. Neocortices were harvested under sterile conditions in a buffer solution containing 137 mM NaCl, 5 mM KCl, 170 µM Na$_2$HPO$_4$, 205 µM KH$_2$PO$_4$, 5 mM glucose, 59 mM sucrose, 100 U/ml penicillin and 0.1 mg/ml streptomycin, pH 7.4. The cortices were minced with scissors and digested using 0.25% trypsin for 5 minutes, then with
addition of 0.016% DNase for a further 5 minutes at 37°C and mixed at 30 rpm. The cortices were centrifuged (400xg, 1600 rpm) for 5 minutes at room temperature, the supernatant was aspirated and the tissue pellet was re-suspended in Gibco® DMEM/GlutaMAX-1 (Invitrogen Corp, Carlsbad, CA) containing 10 mM HEPES, 100 U/ml penicillin, 0.1 mg/ml streptomycin and 10% horse serum, pH = 7.4. The tissue was dissociated by trituration and filtered through a 100-µm Nitex screen. Cells were plated at a density of 50,000 cells/well in 96-well polystyrene plates (Corning, Inc., Corning, NY) that had been pre-coated with poly-L-lysine. Cells were incubated at 37°C in a humidified atmosphere of 5% CO₂ and 95% air.

Multi-compartment pharmacokinetic models for the disposition of deltamethrin and permethrin (Tornero-Velez et al. 2007, Mirfazaelian et al. 2006) were used to predict tissue concentrations of deltamethrin and permethrin in the brain at 6 h following the acute administered doses used in this study. Predictions are listed in Table 2.7. These estimated brain concentrations were then used to select nominal media concentrations of pyrethroids for use in the functional neurite morphogenesis cell model.

For in vitro exposure of cells, pyrethroids were prepared in DMSO using semi-logarithmic serial dilutions of concentrated stock solutions to yield working solutions ranging from 0.001 – 0.03 mM and 0.01 – 3 mM for deltamethrin and permethrin, respectively. On the day of use, working solutions were diluted 1/1000 into the cortical media to produce a final chemical concentration range of 0.001 – 0.03 µM and 0.01 – 3 µM for deltamethrin and permethrin respectively. The final DMSO concentration in the cortical media was 0.1%. Chemicals were added to the cells 2 hours after plating to ensure the cells adhered to the poly-L-lysine and incubated for a 96-hour exposure period.
**Evaluation of neurite outgrowth.** A Neurite Outgrowth Hitkit (Thermo-Fisher Scientific, Waltham, MA) was used to evaluate cortical cell neurite outgrowth and branching. Cortical cells were fixed for 20 min using a 4% paraformaldehyde solution dissolved in PBS at 37°C. Hoechst 33258 dye was included in the fixative to label cell nuclei. Cell bodies and processes were labeled using an anti-βIII tubulin primary antibody, followed by an Alexa Fluor 488-conjugated secondary antibody. Plates were evaluated using a Cellomics ArrayScan VTI high content imaging platform for automated image capture and analysis. A multiple bandpass emission filter and matched excitation filters were used to acquire fluorescence images of the nuclei (blue) and cell body/processes (green) using a high-resolution charge-coupled device (CCD) camera. The acquired images were analyzed using the Neuronal Profiling bioapplication (Thermo Fisher Scientific) to measure cell-based changes in neuronal morphology (i.e., total neurite length, neurite count, branch points, and cell body area). Using a 10X objective, a sufficient number of fields were acquired for the analysis of at least 200 cells per well.

**Cell viability.** Cellular viability was determined in cortical cell cultures grown as described above in opaque 96 well plates using the CellTiter-Glo Viability Assay (Promega Corp., Madison, WI). The number of viable cells in each well was determined through quantification of ATP, an indication of metabolically active cells. Addition of the CellTiter-Glo reagent (100 µl/well) caused cells to lyse and induced a luciferase-catalyzed reaction of ATP with luciferin that produced a luminescent signal proportional to the amount of ATP present. Luminescence in each well was measured thirty minutes after adding the reagent using a FLUOstar Optima plate reader (BMG LABTECH, Durham, NC). Preliminary
experiments verified that ATP content was proportional to the number of live cells in a well (data not shown).

Results.

Microarray dose-response analyses. Both the PIR (isotonic) and SAM (linear) penalized regression methods identified dose dependent increases and decreases in mRNA expression in the cerebrocortex 6 h after an acute, oral exposure to both deltamethrin and permethrin. A comparison the PIR and SAM regression models demonstrate that the two methods yield similar results in terms of identifying dose-responsive probe sets in both the deltamethrin and permethrin treated dose-response cohorts (Figure 2.1A & 2.1B). The rank order of statistical significance was similar between the two methods in that probe sets with test-statistics in the higher end of the observed PIR $M$-statistic range also had test-statistics in the higher end of the observed SAM $d_i$-statistic range. The same trend was observed in the lower end of the test-statistic ranges.

The SAM analyses identified a small number of probe sets with dose-dependent increases in expression following either deltamethrin ($n = 7$) or permethrin ($n = 10$) exposure using the permutation-based false discovery rate values as the significance criteria ($FDR: q < 0.10$, see Figure 2.1A & 2.1B). The PIR analyses did not identify any probe sets for either pyrethroid with dose-dependent changes in expression at this $q < 0.10$ FDR significance threshold. However, the probe sets with $q < 0.10$ in the deltamethrin and permethrin SAM analyses also had $M$-statistics in the extreme upper portion of the observed PIR test-statistic range. Therefore, these methods yield comparable results in that a rank-ordered list of dose-dependent changes in expression constructed using either the PIR or SAM test-statistics
identifies the same groups of probe sets as being the most significantly changed in the
deltamethrin and permethrin test cohorts.

A less statistically conservative method of identifying dose-related changes in probe
set expression identified a larger number significantly altered probe sets than that observed
using the FDR criteria. Both PIR and SAM also calculate an empirical p-value associated
with the regression model for each probe set on the microarray. Using a threshold of \( p < 0.01 \) the SAM analysis identified 70 and 61 probe sets with dose-dependent changes in
expression for deltamethrin and permethrin, respectively, while the PIR analysis identified 93
and 85, respectively (Figure 2.1A-B). In the deltamethrin analyses, 49.5% of all the probe
sets identified had \( p < 0.01 \) for both the SAM and PIR methods (Figure 2.1A, green points).
Likewise, in the permethrin analyses, 53.7% of all the probe sets identified had \( p < 0.01 \) for
both the SAM and PIR methods (Figure 2.1B, green points). Therefore, the overlap between
probe sets identified as dose-responsive using the empirical p-value thresholds is incomplete.
Similar to what is observed using the FDR q-value significance criteria, use of the empirical
p-value significance criteria also demonstrates that probe sets commonly identified using
either the PIR or SAM method tend to appear at the top of a rank ordered list of significance,
as indicated by the convergence of green points in the upper-right and lower-left hand
corners of Figures 2.1A and 2.1B.

To provide a measure of multiple-comparison Type I error control, all the probe sets
for each compound that had empirical p-values < 0.01 in either the SAM or PIR regression
methods were additionally analyzed with a one-way ANOVA with dose as the independent
factor, followed by a Benjamini-Hochberg multiple testing correction (significance threshold,
\( p < 0.05 \)). For deltamethrin and permethrin, 95 of 109 (87.1%) and 53 of 89 (59.5%) probe
sets passed the ANOVA significance threshold. A larger percentage of probe sets (deltamethrin: 12.9 %, permethrin: 44.3 %) identified in the SAM analyses did not pass the ANOVA significance threshold as compared to those identified in the PIR analyses (deltamethrin: 8.7 %, permethrin: 23.8 %). The full list of probe sets considered significantly dose-responsive for deltamethrin (n = 95) and permethrin (n = 53) are given in Tables 2.2 and 2.3. Probe sets listed on these tables that correspond to known protein-coding RefSeq database entries were considered candidates for qRT-PCR confirmation in dose-response Cohorts 3 and 4.

The dose-dependent changes in mRNA expression identified with the above analyses are relatively small in magnitude, < 2-fold change from control. Figure 2.2 illustrates the dose-response patterns detected by the SAM and PIR analyses in terms of fold-change from control. For deltamethrin, the magnitude of fold-changes in expression ranged from 0.51 to 1.96 (Figure 2.2A-C). Similarly for permethrin, fold-change values ranged from 0.54 to 1.56-fold (Figure 2.2D-F). No fold-change exclusion criteria were used to filter gene expression changes in the present study.

Further analysis of the dose-response functions illustrated in Figure 2.2 indicate that significant alterations in mRNA expression occur at doses below those needed to produce acute behavioral effects. For each probe set included in the gene-of-interest lists for deltamethrin (Table 2.2) and permethrin (Table 2.3) a Dunnett’s mean contrast test was performed comparing the mean of each dose condition with the mean of the vehicle treated control group. The cumulative results of these tests are illustrated in the inset panels of Figure 2.2. A majority of the probe sets identified as dose-responsive had mean expression values in the 3 mg/kg deltamethrin and 100 mg/kg permethrin dose groups different from
those in the vehicle treated control group (78.9 % and 77.3 %, respectively). Of those probe sets, 25.3 % and 19.5 % also had mean expression values in the 1 mg/kg deltamethrin and 10 mg/kg permethrin dose groups different from controls. These doses are below those needed to produce acute neurotoxic effects on behavior. In addition, these data demonstrate that the PIR analyses detected a greater number of probe sets with mean expression values in the behavioral “NOAEL level” dose groups (see Table 2.1) as being different from control as compared to the SAM analyses (Figure 2.2B & E, insets).

**Comparison of transcriptional effects across compounds.** A comparison of the probe sets identified as dose-responsive in the PIR and SAM regression analyses demonstrates that the transcriptional response elicited by the two pyrethroids has some common characteristics. The panels in Figures 2.3 plot the $-\log_{10}$ of the empirical $p$-values associated with the PIR regression (2.3A) or SAM regression (2.3B) for each probe set identified as dose-responsive for either deltamethrin or permethrin. Data from the PIR regression analyses demonstrate that expression of 27.2 % of all probe sets identified as dose-responsive for either pyrethroid are significantly altered by both compounds at an empirical $p$-value threshold of $p < 0.05$ (Figure 2.3A). Likewise, SAM analyses demonstrated that 27.8 % of all dose-responsive transcripts are altered by both pyrethroids (Figure 2.3B). Differences in the transcriptional response profiles between pyrethroids are also apparent and may represent divergent biological actions elicited by the different pyrethroids.

**Quantitative real-time RT-PCR.** Measurement of transcript expression by qRT-PCR confirmed the dose-related trends in expression observed during microarray analysis in a
group of nine transcripts taken from Tables 2.2 and 2.3. Table 2.4 summarized the results of these assays and compares them to the fold-change expression values derived from the microarray study. To confirm dose-related changes in transcript expression from the microarray study, a separate cohort of test subjects was dosed under the same conditions as those used previously. This provides both a technical and biological replication of the results observed in the microarray analyses. Given the similarities observed in the transcriptional responses elicited by deltamethrin and permethrin, qRT-PCR data were analyzed with a two-way ANOVA with compound and equipotent dose-level (EDL) as the independent factors \((p < 0.05 \text{ significance threshold})\). Where a significant interaction of compound and EDL or a significant main effect of compound was observed, data were subsequently analyzed by a one-way ANOVA within compound with dose as the independent factor.

Of the nine transcripts examined by qRT-PCR, \(\text{Ca}^{2+}/\text{calmodulin dependent protein kinase 1}\gamma (\text{Camk1g})\) and dopa decarboxylase \((\text{Ddc})\), were commonly affected by both compounds, indicating that for these genes there was no differences in the changes in expression elicited by equipotent doses of either pyrethroid. \textit{Camk1g} mRNA expression was significantly increased with both compounds with expression levels different from control at the three highest equipotent dose levels examined \((\text{Dunnett’s test}, p < 0.05)\). \textit{Camk1g} qRT-PCR expression values closely resembled those observed in the microarray study. \textit{Ddc} mRNA expression was significantly decreased with both compounds in the qRT-PCR assays with significant decreases in expression at the ED\(_{30}\) and ED\(_{50}\) dose levels \((\text{Dunnett’s test}, p < 0.05)\). In contrast to \textit{Camk1g}, the microarray dose-response cohort demonstrated a dose-related change in \textit{Ddc} expression for deltamethrin only even though a clear dose-dependent
decrease in Ddc mRNA expression was observed in both the deltamethrin and permethrin qRT-PCR cohorts.

A significant interaction between compound and EDL was observed for glycerol-3-phosphate dehydrogenase 1 (Gpd1) and FK506-binding protein 5 (Fkbp51), indicating that equipotent doses of the two pyrethroids did not elicit similar changes in expression at 6 hours post-exposure. A main effect of dose was observed for Gpd1 and Fkbp51 mRNA only for deltamethrin (Table 2.4). For both Gpd1 and Fkbp51 the mean expression level in the 3 mg/kg deltamethrin dose groups was different from control (Dunnett’s test, \( p < 0.05 \)). In addition, the qRT-PCR expression values for Gpd1 and Fkbp51 closely match those observed in the microarray study.

The immediate early genes, FBJ murine osteosarcoma viral oncogene homolog (c-fos) and early growth response 1 (Egr1) were differentially affected by the two pyrethroids at 6 h post-exposure, however, no significant main effect of dose (EDL) was observed for either compound. For deltamethrin, the direction of fold-change for c-fos and Egr1 is down in most dose groups measured by qRT-PCR. The expression of c-fos was significantly decreased from control values for 3 mg/kg deltamethrin only. In contrast, for permethrin no change in the expression of c-fos and Egr1 mRNA across dose groups in the qRT-PCR cohort. While c-fos and Egr1 expression at 3 mg/kg deltamethrin and 100 mg/kg permethrin reflect the direction of fold-change observed in the microarray study, very little similarity is apparent between qRT-PCR and microarray expression values at the lower dose levels (Table 2.4).

There were no effects of pyrethroid exposure on mRNA expression for heat shock 27kDa protein (Hsp27), brain derived neurotrophic factor (BDNF) or Ras association (RalGAS/AF-6) domain family 6 (Rassf5). In the case of BDNF, qRT-PCR expression
values closely approximate the expression values observed in a second probe set not identified as dose-responsive in the microarray analyses.

Characterization of the time course of mRNA expression for Camk1g, Gpd1, c-fos and Egr1 demonstrates that altered expression of these transcripts also occurs at times earlier than 6 h following acute, oral pyrethroid exposure. Figure 2.4 illustrates the time course of gene expression changes following exposure to either 3 mg/kg deltamethrin or 100 mg/kg permethrin. A summary of the statistical analyses performed on these data is provided in Table 2.5. Treatment-related increases in Camk1g and Gpd1 mRNA expression were observed for both deltamethrin and permethrin. For deltamethrin, both Camk1g and Gpd1 mRNA had maximally induced expression at 3 h followed by persistent elevations at 6 h (Figure 2.4). Both of these changes reached statistical significance in the time course cohorts (Table 2.5). For permethrin, both Camk1g and Gpd1 had maximal induction at 6 h preceded by slight elevations at 3 h. Permethrin-mediated Gpd1 induction was statistically significant while Camk1g induction reflected the trends observed in the dose-response cohorts but did not reach statistical significance. Ddc mRNA expression was decreased following both deltamethrin and permethrin exposure. For deltamethrin decreased expression began at 6 h and persisted through 9 h while for permethrin, expression decreased at 6 h only.

In the qRT-PCR time course cohorts both deltamethrin and permethrin exposure results in the induction of the immediate early genes c-fos and Egr1, albeit with different temporal characteristics. This is contrary to the trends in c-fos and Egr1 expression observed in the deltamethrin and permethrin dose-response cohorts, where the former pyrethroid produced a trend toward decreased expression while the latter produced a trend toward increased expression. Expression of c-fos and Egr1 increases at 3 h for both deltamethrin
and returns to control levels at 6 h. For permethrin, expression of \( c-fos \) and \( Egr1 \) increases at 3 h, remains persistently elevated at 6 h and returns to control levels by 9 h. Even though the microarray data at 6 h demonstrates opposite trends in \( c-fos \) and \( Egr1 \) expression for deltamethrin and permethrin, the data in Figure 2.4 demonstrate that the two pyrethroids, in fact, elicit similar responses in the expression of some immediate early genes. The expression of other immediate early genes, such as \( BDNF \), is apparently not affected by pyrethroids under the dosing paradigm used in this study.

In summary, the mRNA expression levels measured using microarray compared favorably with those observed using qRT-PCR in most cases. In addition, the dose-dependent trends and time course profiles examined in these qRT-PCR studies for \( Camk1g, Gpd1, Ddc, c-fos \) and \( Egr1 \) are qualitatively similar for both deltamethrin and permethrin.

**Significant Analysis of Function and Expression (SAFE).** Seven GO categories were identified as commonly enriched for both pyrethroids using SAFE analysis and Fisher’s \( \chi^2 \) method (Table 2.6, part I). The composition of the commonly enriched categories for both chemicals included genes involved in neuronal morphogenesis, intracellular \( \text{Ca}^{2+} \) signaling and small molecule transport. In addition, five GO-BP categories and two canonical KEGG pathways were identified as enriched in the individual SAFE analyses of permethrin and deltamethrin, respectively (Table 2.6, part II). For permethrin, the SAFE findings include enriched gene categories related to neuronal morphogenesis and developmental patterning. For deltamethrin the SAFE findings include two KEGG metabolic pathways, one of which involves synthesis of the precursor molecules for monoamine neurotransmitters.
SAFE plots of the GO categories ‘morphogenesis of a branching structure’ and ‘Ca+2/calmodulin dependent protein kinase complex’ demonstrated significant category enrichment for both permethrin and deltamethrin (Figure 2.5). This is evidenced by the divergence of the stair step line from the unity line near the far left of Figure 2.5, panels A-D. A SAFE plot of a GO category not significantly enriched for either compound is given in Figure 2.5, panels E-F for comparison purposes. The most significantly dose-responsive transcripts for each of the enriched GO categories are illustrated in the heatmaps to the right of Figure 2.5, panels A-D. These heatmaps demonstrate that appreciable dose-dependent increases in the expression of probe sets contained within the enriched GO categories occurs following pyrethroid exposure. The functional category level analysis of the microarray data provided the basis for testing the hypothesis that pyrethroids affect neuronal branching morphogenesis.

Pyrethroid effects on neurite length and branching in primary mixed cortical cell cultures.

Both deltamethrin and permethrin produce an increase in the number of neurite branch points following a 96 h exposure (Figure 2.6A & D). The range of predicted tissue concentrations (in µM) from the PK predictions (Table 2.7) are marked near the x-axes and correspond well to areas along the in vitro dose-response curve where changes in branching were observed. An average increase of ~25% above control in the number of neurite branch points was observed at nominal media concentrations ranging from 0.01 - 0.03µM deltamethrin and 0.01 – 3µM permethrin. No significant increase in total neurite length was observed for either compound save at the 0.01µM exposure level for permethrin. Changes in cell viability were not apparent in the concentration ranges tested.
Discussion.

A principal finding of the present study was that acute, oral pyrethroid exposure caused dose-dependent alterations in gene transcription in the cortex at doses of deltamethrin and permethrin that were below those required to elicit acute neurotoxic effects in the whole animal (Wolansky et al. 2006). Both similarities and differences in the overall transcriptional response were observed when comparing the two pyrethroids. Quantitative real-time RT-PCR analysis in additional cohorts of animals provided an independent biological and technical replicate of the findings from the microarray data set. In addition, transcripts for which the time course of gene expression was characterized demonstrated qualitative similarities in the response for both pyrethroids. Finally, SAFE analysis of the microarray data identified several GO categories jointly enriched by both deltamethrin and permethrin including some related to branching morphogenesis. Subsequently, a significant increase in the number of neurite branch points was observed in a primary cortical cell culture model. The transcriptional and functional changes observed in this study are consistent with the downstream responses expected to occur following perturbation of normal neuronal firing patterns (Majdan and Shatz 2006; Xiang et al. 2007) such as those that may be produced by pyrethroid-mediated effects on ion channel function.

The following sections include interpretations of the microarray analysis findings, the data analysis framework, a discussion of how some of the individual transcripts identified in this study may be regulated by the pharmacological actions of pyrethroids, some hypothesis of how changes in the expression of these genes may contribute to pyrethroid neurotoxicity and finally a discussion of the pyrethroid effects on neuronal morphology.
Microarray dose response analyses. Dose-dependent alterations in transcript expression were observed in the frontal cortex at 6 h following acute exposure to pyrethroids. In the present study Affymetrix GeneChips® were used as a discovery tool for identifying dose-dependent changes in gene transcription. The PIR and SAM penalized regression models used in this study provided qualitatively similar outcomes in terms of identifying dose-related alterations in gene expression. The two penalized regression analyses demonstrated that both methods ranked a similar sub-set of transcripts as being the most dose-responsive for each compound (Figure 2.1A & 2.1B). Prior to experimentation, the shape of the dose-response curve for any potential alterations in gene transcription was unknown. The SAM regression model, which was designed to test if dose-response curves are fitted by a linear function, detected a number of dose-dependent changes in gene transcription. However, Figure 2.2 clearly illustrates the heterogeneity of the transcriptional dose-response functions observed in the present study. Not all of these functions are adequately fitted by a linear model. A penalized isotonic regression (PIR) analysis developed from Hu et al. (2005) that searches for monotonic increases or decreases in expression without testing the fit to a particular dose-response function, identified dose-related changes in expression that were not detected by SAM. A majority of these had significant increases in expression from control at the “NOAEL” dose of the test compounds. While both SAM and PIR identified the same subset of transcripts as being the most-significantly dose-responsive within each compound (Figure 2.1A & B), the PIR method provided superior detection of probe sets with treatment related changes in expression that did not fit a linear model.

Collectively, these data indicate that the pyrethroid dose thresholds required to elicit changes in gene expression vary from gene to gene and that the regulation of some genes
occur at lower pyrethroid doses than others. For some transcripts, appreciable alterations in mRNA expression occurred exclusively at the highest administered dose of either pyrethroid compound. For other transcripts, changes were observed at doses not associated with any observable neurotoxic effects (Figure 1.2 insets and Table 2.4). The biological factors mediating the heterogeneity in dose-response functions for different pyrethroid-sensitive transcripts are unclear but could be due to several factors. In neurons, induction or repression of gene expression is controlled by a large and diverse network of intracellular signaling cascades that control the activity of numerous transcription factors in the cell nucleus. These neuronal signaling pathways are activated by either: 1) changes in cytoplasmic second messenger levels in response to activation of voltage-sensitive ion channels, ionotropic or metabotropic neurotransmitter receptors or membrane bound receptor complexes or 2) exogenous ligands that are transported into the cell (such as glucocorticoids) to directly enhance transcription factor activity (de Kloet 1998; West et al. 2002; Schulman and Roberts 2003; Schoneveld 2004). Some of these signal transduction pathways may become activated at lower administered doses of pyrethroids than others, thereby causing the transcripts they control to become induced or repressed at different points along the administered dose range. Alternatively, the use of only one time point in the dose-response study may have contributed to the differences in dose-responsive expression patterns between genes. Differences in the duration of signaling pathway stimulation or transcription factor activation and the rate of mRNA synthesis and degradation may result in variable temporal expression patterns between pyrethroid-sensitive genes.

Time course data demonstrate that pyrethroid-sensitive genes have varying temporal profiles of expression (Figure 2.4). The sampling time of 6 h in the initial phase of this study
was selected to capture both ‘early’ and ‘late’ transcriptional changes in response to pyrethroid exposure. The time course data at 6 hr are consistent with the data from the dose-response study which was collected 6 h after exposure. In addition, the available time course data implies that additional alterations in gene expression not detected in the dose-response study may occur at time points other than the one sampled. The qRT-PCR data shown here support this conclusion (Figure 2.4).

In the present experiment, the cortex was selected as the tissue-of-interest based on previous studies demonstrating rapid accumulation of both deltamethrin and permethrin following an acute oral dose, disruption of cortical neuronal firing patterns, and induction of gene products known to be upregulated following neuronal excitation (Ray 1980; Anadon 1991; Hassouna et al. 1996; Anadon 1996; Condes-Lara 1999). However, the use of a brain tissue that is a heterogenous mixture of neuronal sub-types (e.g. glutaminergic, GABAergic), glial cells and vascular endothelia (Hof et al. 2003) presented a particular challenge in analysis of the microarray data: namely, analysis of transcripts with low relative abundance in the total RNA sample. One result of this cellular heterogeneity is lower relative fluorescent signal intensities (and therefore lower expression summaries) of neuron-specific transcripts in the microarray data set than what would be observed using a total RNA sample from an exclusively neuronal cell population (c.f. Ginsberg and Che 2005). Effectively, the neuronal mRNA is diluted by mRNA from other cell types. Arbitrary exclusion of lowly expressed mRNAs from the analysis based on a fluorescence threshold may unduly discard probe sets that have biologically relevant changes in expression. To control for lowly expressed genes without excluding potentially meaningful data from the analysis, both the SAM and PIR penalized regression methods use an adjustment factor in the calculation of the
test-statistics to force probe sets with very low signal intensities to change more dramatically to be considered significant (Tusher et al. 2001; Hu et al. 2005). In the present study the penalized regression analyses reduced the number of lowly-expressed, and potentially unreliable, probe sets included in the dose-responsive gene-of-interest list (Appendix A, Figure 1) while also provided a means of surveying the entire data set for pyrethroid-responsive genes.

The magnitude of the transcriptional alterations (expressed as fold-change from control) observed in the present study are comparable to those observed in other microarray studies that examine the acute effects of pharmacological agents on gene transcription in the brain (Li et al. 2004; Xie et al. 2004; Dow et al. 2005; Bowyer et al. 2007). The relatively small number of significant changes in expression was not unexpected given the nature of the pyrethroid exposures used: i.e. even the highest doses used in this study were only slightly above the threshold needed to elicit acute behavioral effects (Bloom et al. 1983; Peele and Crofton 1987; McDaniel and Moser 1993; Wolansky et al. 2006). The low doses and resulting small magnitude transcriptional changes observed in this study are likely the reasons why only a very small number of transcripts were identified as dose-responsive using the permutation based FDR estimates as the significance criteria ($q < 0.01$). In analyses of training data sets, it has been demonstrated that the SAM analyses can efficiently detect treatment related effects in transcripts with changes in expression ($> \sim 1.5$-fold) by using the permutation-based FDRs as a significance threshold (Tusher et al. 2001; Larsson et al. 2005). However a systematic analysis of how well these permutation methods perform in picking up small magnitude gene expression changes has not been performed.
Comparison across compounds. The present data demonstrate both similarities and differences in the global transcriptional response in rat cortex to acute, low-dose deltamethrin and permethrin exposure (Figure 2.3). Of the transcripts examined by qRT-PCR (Table 2.4 and Figure 2.4), Camk1g, Ddc, Gpd1, c-fos and Egr1 all had similar expression profiles for both deltamethrin and permethrin. These similarities were not readily apparent upon examination of the microarray probe set data (Cohorts 1 & 2) or 6 h qRT-PCR data (Cohorts 3 & 4). For instance, the immediate early genes c-fos and Egr1 were identified as dose-responsive for permethrin during microarray analysis and had opposite trends in expression at 6 h for the two pyrethroids. Similarly, Camk1g and Gpd1 were identified during microarray analysis for deltamethrin, but not permethrin, even though a similar trend in the direction of change is observed across compounds. Characterization of the time course of mRNA expression for these genes clearly demonstrates a commonality in the transcription responses elicited by the two compounds. The results of the SAFE functional category level analysis support the conclusion that the biological activities of the two pyrethroids overlap. Several categories were found to be commonly upregulated between the two compounds. How many of these individual gene changes or impacted functional categories are relevant to the neurotoxicology of pyrethroids remains yet to be determined. These data provide guidance on some novel cellular functions affected by pyrethroids.

Biological significance of experimental findings. Interestingly, probe sets corresponding to the primary molecular targets for pyrethroids were not altered for either pyrethroid tested in the microarray study. Specifically, there were no treatment related changes in any of the VSSC or VSCC isoforms / subunits or any subunits that comprise neurotransmitter receptors.
complexes (Cull-Candy et al. 2001; Ogata and Ohishi 2002; Catteral et al. 2003; Farrant and Kalia 2007). This finding is supported by an *in vitro* study by Xiang et al. (2007) that used a variety of pharmacological manipulations to increase the firing rates of cultured neuronal networks from rat cortex and examined the global transcriptional response of those neurons following stimuli. No changes in the expression of VSSC or VSCC isoforms / sub-units or neurotransmitter receptors were identified in that study in response to increases in neuronal firing rates. The Xiang et al. (2007) study did detect increases in the expression some voltage-sensitive K\(^+\) channels: *Kcnal* and *Kcnk12*. These particular K\(^+\) channel sub-types were not altered in the present study, however *Kcnfl* was upregulated following pyrethroid exposures. The present data do not rule out the possibility that pyrethroids cause transcription-independent changes in the expression or functional state of ion channels or neurotransmitter receptor complexes. Such responses are known to occur in neurons in response to excitatory or depolarizing stimuli (Paillant et al. 1996; Giraud et al. 1998; Shiraishi et al. 2001; Kim et al. 2007). The present data do not support transcriptional induction or repression of VSSCs, VSCCs or neurotransmitter receptor subunits as a neuronal response to pyrethroids.

The immediate early transcription factors *c-fos* and *Egrl* were upregulated following both deltamethrin and permethrin exposure and are in agreement with previous studies of IEG expression in the cortex following acute pyrethroid exposure (Hassouna et al. 1996; Liu and Wu 2001). Increased *Egrl* and *c-fos* expression supports the conclusion that neurons in the cortex were activated by deltamethrin and permethrin in the present study. *Egrl* and *c-fos* are among the genes induced by increased neuronal firing in the Xiang et al. (2007) study as well as in other paradigms for studying neuronal activity (Zangenehpour et al. 2002; Patra...
et al. 2004). Immediate early gene (IEG) induction is known to be the most rapid transcriptional response of neurons following increased activity (Steward et al. 1998; Dassesse et al. 1999; Guzowski et al. 1999; Clayton 2000). The time course for the expression of the IEGs c-fos and Egr1 does not support de novo gene transcription as being responsible for mediating the acute behavioral effects of pyrethroids. The earliest time that increased IEG expression is observed in the present study is at 3 h. IEG expression is at control levels at 1 h. Onset of behavioral effects occurs prior to the onset of increased IEG expression (i.e. 30 min – 1 h, Crofton and Reiter 1984). Therefore, the novel gene expression changes described herein can not mediate the acute neurotoxic signs of pyrethroid intoxication.

An increase in the expression Ca\(^{2+}\)/calmodulin dependent protein kinase 1g (Camk1g) mRNA was also observed following deltamethrin and permethrin exposure in vivo. There is evidence in the literature that Camk1g mRNA expression is regulated by changes in neuronal firing patterns similar to IEGs. In the Xiang et al. (2007) study increases and decreases in the expression of Camk1g mRNA matches nearly perfectly with increases and decreases in neuronal burst firing rates. Increases in Camk1g mRNA expression have been reported in response to acute or chronic exposure to the opioid analgesic morphine in mouse striatum and chronic exposure to the atypical antipsychotic olanzapine in the rat frontal cortex (Fatemi et al. 2006; Korostynski et al. 2007). Both of these agents are known to increase spontaneous and/or stimulus evoked bursting activity in some brain regions in vivo (Stanzione et al. 1988; Gronier and Rasmussen 2005) further supporting that Camk1g mRNA expression is correlated with changes in neuronal activity. Therefore, increased Camk1g mRNA expression
expression observed in the present study is most likely due to pyrethroid-induced alterations in neuronal firing rates within the frontal cortex.

Increased expression of *Camk1g* may impact on the structure and function of pyrethroid affected neurons. This is consistent with recent data from *in vitro* models of developmental neurite and dendrite morphogenesis. Wayman et al. (2006) demonstrated that *Camk1g* plays a specific role in the activity-dependent growth of hippocampal neurons between 7-9DIV by mediating activation of a Ras/MEK/ERK signaling cascade in response to excitatory stimuli and triggering CREB-mediated transcription of *Wnt-2*, a soluble autocrine factor that promotes dendritic growth. Takemoto-Kimura et al. (2007) demonstrated that *Camk1g* is covalently modified, inserted into lipid rafts and targeted to neuronal dendrites where it activates a Rac signaling pathway that mediates the morphogenesis of cortical neurons. In both the Wayman et al. (2006) and Takemoto-Kimura et al. (2007) studies, knockdown or overexpression of *Camk1g* altered depolarization and neurotrophin-stimulated outgrowth of neuronal processes, respectively. Takemoto-Kimura et al. (2007) also demonstrated that *Camk1g*-mediated effects are restricted to dendrites (axons were not affected). The role of *Camk1g* in maintenance and activity-regulated plasticity of dendritic arbors in adults (Sin et al. 2002) is currently unknown. Given this knowledge of *Camk1g* activity *in vivo*, it is plausible that pyrethroid-mediated increases in *Camk1g* expression (especially in a developmental context) could result in changes in neuronal morphology. In the present study, *in vitro* exposure of developing cortical neurons to both deltamethrin and permethrin resulted in an increase in the number of neurite branch points. The role of *Camk1g* induction in mediating these effects remains unknown.
The transcriptional upregulation of glycerol-3-phosphate dehydrogenase 1 (*Gpd1*) and FK506-binding protein (*Fkbp51*) mRNA in the present study has two major implications: 1) that *in vivo* pyrethroid exposure results in the activation of hypothalamic-pituitary-adrenal (HPA) axis and 2) that the non-neuronal cell populations in the brain are sensitive to pyrethroids, most likely through an indirect activation of the HPA axis. The expression of *Gpd1* mRNA was transiently increased following deltamethrin and permethrin exposure (Table 2.4 and Figure 2.4). Similarly, expression of *Fkbp51* mRNA dose-dependently increased at 6 h following deltamethrin exposure only (Table 2.4), although this transcript was not examined in the time course cohort. These changes in *Gpd1* and *Fkbp51* mRNA expression suggest that pyrethroids can have effects on glial cell populations as the proteins encoded by these transcripts are expressed in the brain exclusively in oligodendrocytes (Leveille et al. 1980) and T-cell lymphocytes (Baughman et al. 1995). However, the induction of these mRNAs is likely an indirect action of pyrethroids via activation of the HPA axis (de Kloet et al. 1998). Both the *Gpd1* and *Fkbp51* genes contain glucocorticoid receptor binding motifs either in the upstream promoter region (*Gpd1*, Cheng and de Vellis 2000) or in an intronic region (*Fkbp51*; Hubler and Scammell 2004) and increased expression of both is dependent upon glucocorticoid hormone stimulation (Nicols et al. 1996; Baughman et al. 1997). Glucocorticoids are released in the circulation from the adrenals in response to a variety of stressors. Interestingly, increased circulating corticosterone levels were reported in the rat following deltamethrin exposures; albeit at very high, intravenous doses (de Boer et al. 1998). Increases in *Gpd1* and *Fkbp51* expression may be considered to be components of a generalized, non-specific stress response brought about by overstimulation of the HPA axis.
by pyrethroids. The potential impact of increased Gpd1 and Fkbp51 expression on the health and function of affected glia, to date, is unclear.

Data from the present study suggest that the pathways controlling monoaminergic neurotransmitter synthesis may be affected by pyrethroids. Both deltamethrin and permethrin caused decreases in the expression of aromatic L-amino acid decarboxylase (Ddc) mRNA in the frontal cortex at 6 h (Table 2.4). Small differences in the time course of Ddc mRNA expression between the compounds were observed with deltamethrin causing a more persistent decrease than permethrin. Ddc is the final enzyme in the synthesis pathways of dopamine and serotonin, neurotransmitters derived from the amino acids tyrosine and tryptophan, respectively (Deutch and Roth 2003). This is consistent with previous reports noting a depletion of dopamine and serotonin in a variety of brain regions following repeated exposure to deltamethrin (Martinez-Larranaga et al. 2003; Liu et al. 2006; Liu and Shi 2006). In the case of dopamine depletion, two of these studies demonstrate concurrent decreases in the expression of tyrosine hydroxylase, the penultimate enzyme in dopamine synthesis (Liu et al. 2006; Liu and Shi 2006). Expression of Ddc was not examined in these studies.

The increased expression of c-fos, Egr1 and Camk1g in the present study are most likely regulated by pyrethroid-induced changes in the neuronal firing patterns of cortical neurons. In contrast, the increased expression of Gpd1 and Fkbp51 mRNA indicates that the HPA-axis was activated by pyrethroid exposure indicating an excitatory effect of these compounds on the sympathetic nervous system. Previous studies have utilized transient activity-dependent alterations in gene expression to map out activated neuronal circuits in response to excitatory or neurotoxic stimuli (Carvajal et al. 2005; Guzowski et al. 2005; Guzowski 2006) essentially using the expression of these mRNA species as biomarkers of
pharmacological or excitatory actions at the neuronal membrane. The data from this study indicate that *c-fos, Egr1* and *Camk1g* may be appropriate for use in this type of functional mapping in examination of the pharmacological actions of pyrethroids.

**Pyrethroid effects on branching morphogenesis.** Both deltamethrin and permethrin increased the number of neurite branch points in an *in vitro* model of cortical neuronal development with no effects on total neurite length. The pharmacokinetic modeling data show these effects occurred at media concentration ranges comparable to tissue concentrations predicted to be present in the brain following an acute exposure *in vivo* (Table 2.7, Figure 2.6). This confirms the results from the functional category (SAFE) analysis which predicted that branching morphogenesis would be affected by pyrethroids. This was based on dose-dependent patterns of gene expression for mRNA transcripts that code proteins which have been shown to control neurite branching and morphogenesis in the nervous system. These genes include *Cxcl12, Notch1, β*-catenin and *Camk1g* (Redmond et al. 2000; Yu and Malenka 2003; Pujol et al. 2005; Wayman et al. 2006; Takemoto-Kimura et al. 2007). A majority of the studies concerning the function of this group of genes focused on their role in developmental neuronal morphogenesis. Therefore, a development model of neurite growth was used to assess pyrethroid effects on neuronal branching. The gene expression data from the present study support that pyrethroid effects on neurite branching and not length may be due to increased expression of a suite of genes that shifts the growth pattern of neurons away from extension of neuronal processes and more towards neurite branching. Overexpression of *Notch1* in rat cortical neurons results in an increase in neuronal branching and an antagonism of neurite extension (Redmond et al. 2000).
Likewise, overexpression of β-catenin and Cxcl12 results in increased dendritic and axonal branch tip number, respectively and has no or opposite effects on measures of length (Yu and Malenka 2003; Pujol et al. 2005). All of these transcripts are upregulated following pyrethroid exposure in vivo (see Figure 2.5 heatmaps). The exact cellular mechanisms that mediate pyrethroid-induced increases in branching are currently unknown.

The present data support that pyrethroids affect the developmental morphogenesis of neurons. However, these data are not consistent with the results of previous studies of pyrethroid effects on developmental neuronal outgrowth. Treatment of developing X. laevis neurons with 10 nM deltamethrin resulted in an increase in total neurite length in the presence of extracellular Ca$^{+2}$ (Lautermilch and Spitzer 2000). In contrast, exposure of N2A neuroblastoma or C6 glioma cells the pyrethroid cypermethrin resulted in no effect on morphology (Flaskos et. al 2007). The disparity between the results from these studies and the present study could be due to differences in the compounds tested, exposure conditions and cell types. Preliminary experiments in PC-12 neuroblastoma cells (data not shown) did not demonstrate any effects on neurite branching or length. This supports the observations of Flaskos et al. (2007) and indicates a disparity between different cell culture models for detecting pyrethroid effects on neuronal morphology. The present data is the first to demonstrate an effect of pyrethroids on the branching morphology of primary cultured neurons.

Disruption of neuronal morphogenesis in the developing nervous system by pyrethroids could result in detrimental effects on neurological function later in life. Intermittent exposure to stimulant drugs such as amphetamine can produce an increase in dendritic branching in vivo in both juvenile and adult rats (Robinson and Kolb 2004; Diaz
Heijtz et al. 2003; Robinson and Kolb 1999). These morphological changes are thought to underlie some of the adverse neurological effects associated with abuse of stimulant drugs (e.g., learning deficits) (see Robinson and Kolb 2004; Gonzalez et al. 2005). In addition, lead exposure during development results in neurological deficits that have been associated with changes in neuronal morphology (Petit and LeBoutillier 1979; McConnell and Berry 1979; Costa et al. 2004). Both lead and stimulant drugs facilitate neurite outgrowth in \textit{in vitro} cell culture models that is similar, but not identical, to the increased branching observed with pyrethroids in the present study (Kern et al. 1993; Audesirk and Cabell 1999; Crumpton et al. 2001; Park et al. 2002; Park et al. 2003). Collectively, these works demonstrate that \textit{in vitro} models of neuronal outgrowth are sensitive to neurotoxic agents that produce neurological deficits and consequent changes in neuronal morphology \textit{in vivo}. Several questions remain to be addressed before definitive conclusions regarding pyrethroid effects on neuronal morphogenesis can be made, including: 1) whether or not pyrethroid-induced changes in morphology occur \textit{in vivo}, 2) are effects on morphogenesis specific to cortical neurons, and 3) do all compounds in the pyrethroid class produce the same types of effects on neuronal branching morphogenesis?

In conclusion, the present study has identified a group of genes whose transcription is altered in a dose-dependent manner in the rat cortex following \textit{in vivo} pyrethroid exposure. A majority of the gene expression changes observed in this study are consistent with the induction of neuronal hyperexcitability by pyrethroids. The gene expression changes observed are transient, comparable between the two pyrethroids tested and provide insight into the cellular response of the neurons downstream of the pharmacological effects of these compounds at the neuronal membrane. Most importantly, this study provides evidence that
branching of cortical neurons is increased by pyrethroids, suggesting that neurotoxic action of these compounds includes effects on neuronal morphology.

**Supplementary Data.**

Appendix A, Table 1 compares coefficients of variation for probe set expression summaries using either GCOSv1.2 or RMA. Appendix A, Table 2 provides assay identification numbers, context sequences, amplicon lengths and calculated amplification efficiencies for Applied Biosystems TaqMan® qRT-PCR assays used in this study. Appendix A, Figure 1 is a comparison of rank order of significance between SAM-based linear regression and Benjamini-Hochberg adjusted ANOVA analysis methods.

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Table 2.1. *Group sizes of cohorts used in this study.*

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<th>Dose (mg/kg) :</th>
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<th>Deltamethrin</th>
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<td>Vehicle Control</td>
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<td>3</td>
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**Microarray Dose Response**<sup>a</sup>
- Cohort 1: 6
- Cohort 2: 6

**qRT-PCR Dose-Response**<sup>b</sup>
- Cohort 3:
  - 7
  - 7

**qRT-PCR Time Course**<sup>c</sup>
- Cohort 4:
  - 8<sub>4</sub>
  - 8<sub>4</sub>

<sup>a</sup>Microarray data from Cohorts 1 & 2 were combined (n=8/treatment group) with control values from cohorts 1 & 2 (n=12) for dose-response analysis of permethrin and deltamethrin mediated effects, respectively.  
<sup>b</sup>Test animals in Cohort 3 were split into two dose-response studies for permethrin and deltamethrin, respectively, for qRT-PCR confirmation of gene expression changes observed during the microarray study.  
<sup>c</sup>Test animals in Cohort 4 were used for qRT-PCR time course studies. Four time points (1,3,6,9 hours: n = 8/treatment group) per compound with time matched controls (n = 8/control group).
Table 2.2. List of probe sets with dose-dependent changes in expression for deltamethrin. Affymetrix probe set IDs without a gene symbol are expressed sequence tags (ESTs). Probe sets with arrows correspond to genes examined by qRT-PCR. Positive SAM $d_i$ or PIR M scores denote upregulated probe sets. Negative SAM $d_i$ or PIR M scores denote downregulated probe sets.

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Table 2.3. List of probe sets with dose-dependent changes in expression for permethrin. Affymetrix probe set IDs without a gene symbol are expressed sequence tags (ESTs). Probe sets with arrows correspond to genes examined by qRT-PCR. Positive SAM \(d_i\) or PIR \(M\) scores denote upregulated probe sets. Negative SAM \(d_i\) or PIR \(M\) scores denote downregulated probe sets.

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Bold values are 2^ΔΔCT values (± SE) from qRT-PCR Cohorts 3 (DLT) & 4 (PERM). Values in italics are fold-change from control (± SE) for microarray probe sets (Cohorts 1 & 2) corresponding to the transcript of interest. All gene expression measurements were at 6 h following pyrethroid exposure. "V" = Vehicle control. "EDL" = Equivalent Dose Level, defined from Wolansky et al (2006) – see methods section. Main effects in two-way ANOVA: RED = EDL (p < 0.05) and COMPOUND*EDL (p > 0.05), GREEN = COMPOUND (p < 0.05) and COMPOUND*EDL (p > 0.05), BLUE = COMPOUND*EDL (p < 0.05) and main effect of DOSE in a one-way ANOVA for deltamethrin. *= Significant difference from vehicle control in a Dunnett’s mean contrast test (p < 0.05).
Table 2.5. *Two-way analysis of variance (ANOVA) for qRT-PCR time course data.*

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<td>1.42</td>
<td>0.2465</td>
</tr>
<tr>
<td>Ddc</td>
<td>TRT</td>
<td>4.48</td>
<td>0.0387</td>
<td>0.01</td>
<td>0.9419</td>
</tr>
<tr>
<td></td>
<td>TIME</td>
<td>1.78</td>
<td>0.1620</td>
<td>1.01</td>
<td>0.3970</td>
</tr>
<tr>
<td></td>
<td>TRT*TIME</td>
<td>2.17</td>
<td>0.1025</td>
<td>0.91</td>
<td>0.4438</td>
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<tr>
<td>c-fos</td>
<td>TRT</td>
<td>5.78</td>
<td>n/a</td>
<td>10.66</td>
<td>0.0019**</td>
</tr>
<tr>
<td></td>
<td>TIME</td>
<td>6.35</td>
<td>n/a</td>
<td>2.99</td>
<td>0.0386**</td>
</tr>
<tr>
<td></td>
<td>TRT*TIME</td>
<td>6.26</td>
<td>0.0010**</td>
<td>1.56</td>
<td>0.2014</td>
</tr>
<tr>
<td>Egr1</td>
<td>TRT</td>
<td>7.63</td>
<td>n/a</td>
<td>26.12</td>
<td>n/a</td>
</tr>
<tr>
<td></td>
<td>TIME</td>
<td>10.43</td>
<td>n/a</td>
<td>8.26</td>
<td>n/a</td>
</tr>
<tr>
<td></td>
<td>TRT*TIME</td>
<td>7.62</td>
<td>0.0002**</td>
<td>8.40</td>
<td>0.0001**</td>
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<td>BDNF</td>
<td>TRT</td>
<td>0.14</td>
<td>0.7110</td>
<td>2.04</td>
<td>0.1587</td>
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<td>0.8358</td>
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<td>TRT*TIME</td>
<td>0.22</td>
<td>0.8814</td>
<td>0.69</td>
<td>0.5633</td>
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</table>

Two-way ANOVA factors are: TIME, TRT, TIME*TRT. Analysis of 2-\(\Delta\Delta CT\) values is presented. n.e. = no effect. n/a = not applicable. ** = significant effect at \(p < 0.05\). Pair-wise comparisons are within time for a main effect of treatment \((p < 0.05)\).
Table 2.6. Significant Analysis of Function and Expression (SAFE).

<table>
<thead>
<tr>
<th>Category L.D. and name</th>
<th>size*</th>
<th>DLT p-value</th>
<th>PERM p-value</th>
</tr>
</thead>
</table>

**Part I. Commonly enriched gene categories for both permethrin and deltamethrin‡**

**GO Biological Process**
- GO:0048754, ‘branching morphogenesis of a tube’ 6 0.0171 2.00E-04
- GO:0001763, ‘morphogenesis of a branching structure’ 67 0.0172 2.00E-04
- GO:0007162, ‘negative regulation of cell adhesion’ 27 0.0175 0.0025
- GO:0015718, ‘monocarboxylic acid transport’ 30 0.0051 0.0125
- GO:0007498, ‘mesoderm development’ 57 0.0105 0.0067

**GO Cellular Component**
- GO:0005954, ‘calcium- and calmodulin-dependent protein kinase complex’ 25 0.0053 0.0146

**GO Molecular Function**
- GO:0046915, ‘transition metal ion transporter activity’ 44 0.0026 0.0348

**Part II. Enriched gene categories identified by SAFE.**

**Deltamethrin**

**KEGG Pathway**
- KEGG:00564, ‘Glycerophospholipid metabolism’ 73 0.0404
- KEGG:00400, ‘Phenylalanine, tyrosine and tryptophan biosynthesis’ 12 0.0928

**GO Biological Process**
- GO:0048754, ‘branching morphogenesis of a tube’ 66 0.0349
- GO:0001763, ‘morphogenesis of a branching structure’ 67 0.0349
- GO:0001569, ‘patterning of blood vessels’ 31 0.0406
- GO:0009880, ‘embryonic pattern specification’ 49 0.0554
- GO:0045655, ‘regulation of monocyte differentiation’ 32 0.0932

**Permethrin**

- GO:0048754, ‘branching morphogenesis of a tube’ 66 0.0349
- GO:0001763, ‘morphogenesis of a branching structure’ 67 0.0349
- GO:0001569, ‘patterning of blood vessels’ 31 0.0406
- GO:0009880, ‘embryonic pattern specification’ 49 0.0554
- GO:0045655, ‘regulation of monocyte differentiation’ 32 0.0932

‡ = GO categories or KEGG pathways with p < 0.1 for both test compounds using SAFE & Fisher’s $\chi^2$ method. * = number of Affymetrix probe sets included in GO category or KEGG pathway groupings. ** = GO categories or KEGG pathways with an adjusted p < 0.1 for SAFE method.
Table 2.7. Pharmacokinetic estimates of pyrethroid brain concentrations.

<table>
<thead>
<tr>
<th>Administered Dose (mg/kg)</th>
<th>Time (h)</th>
<th>Brain Concentration (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deltamethrin\textsuperscript{a}</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.3</td>
<td>6</td>
<td>0.005</td>
</tr>
<tr>
<td>1</td>
<td>6</td>
<td>0.0169</td>
</tr>
<tr>
<td>3</td>
<td>6</td>
<td>0.050</td>
</tr>
<tr>
<td>Permethrin\textsuperscript{b}</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>6</td>
<td>0.060</td>
</tr>
<tr>
<td>10</td>
<td>6</td>
<td>0.582</td>
</tr>
<tr>
<td>100</td>
<td>6</td>
<td>5.940</td>
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</tbody>
</table>

\textsuperscript{a}Estimates based on Mirfazaelian et al. (2006).
\textsuperscript{b}Estimates based on Tornero-Velez et al. (2007).
Figure 2.1. Comparison of PIR and SAM regression methods. Panels A & B plot the penalized isotonic regression (PIR) test statistic ($M$, $x$-axis) against the penalized linear regression (SAM) test statistic ($d_i$, $y$-axis) for deltamethrin and permethrin, respectively. All 31,042 probe sets present on the Affymetrix Rat 230 2.0 array are shown. Data points in green have an empirical $p$-value < 0.01 for both the PIR and SAM methods. Data points in blue have an empirical $p$-value < 0.01 for the PIR regression only. Data points in orange have an empirical $p$-value < 0.01 for the SAM regression only. Data points circled in red have a $q$-value < 0.10 in permutation-based FDR calculations employed in the SAM algorithm.
**Figure 2.2. Dose-response functions identified by PIR and SAM regression methods.** Panels A-F plot dose-response functions for probe sets identified by PIR (B & E), SAM (C & F) or both regression methods (A & D) for deltamethrin (A-C) and permethrin (D-F). Only probe sets that had a Benjamini-Hochberg adjusted $p$-value $< 0.05$ for a main effect of dose in a one-way ANOVA are shown. For each probe set expression summaries for each treatment group were normalized to vehicle control and plotted as fold-change from control. The color scheme corresponds to that used in Figure 1, with green curves being detected by both PIR and SAM regression methods, blue curves being detected exclusively with the PIR method and orange curves being detected exclusively with the SAM method. Insets on each panel are the summated results of a Dunnett’s many-to-one mean contrast test performed within each probe set comparing the means of the lowest (C-L), middle (C-M) and highest (C-H) doses to the mean of vehicle treated control. $y$-axis is number of probe sets identified under each comparison at a significance level of $p < 0.05$. Note the green portion of the stacked bars in the insets are the same values for panels A-C and D-E, respectively.
Figure 2.3. *Comparison of probe sets identified by PIR or SAM between pyrethroids.* Panels A and B plot the –log$_{10}$ (empirical $p$-value) for deltamethrin (x-axis) against the –log$_{10}$ (empirical $p$-value) for permethrin (y-axis) for probe sets identified during PIR or SAM regression analyses, respectively. All probe sets that had a Benjamini-Hochberg adjusted $p$-value < 0.05 for a one-way ANOVA for either permethrin or deltamethrin are included in the plot. Dashed boxes represent $p < 0.05$, $p < 0.005$, and $p < 0.0005$ empirical $p$-value thresholds. All points in the upper right of the figures, within the dashed boxes, meet the respective $p$-value criteria for both pyrethroids. 27.2% and 27.8% of all probe sets identified during PIR or SAM analysis, respectively, had empirical $p$-values of $p < 0.05$ for both compounds.
Figure 2.4. *qRT-PCR time course results.* Transcript expression over time following a single acute dose of 3 mg/kg deltamethrin (top) or 100 mg/kg permethrin (bottom). Gene symbols are listed on the y-axis. Data were analyzed using two-way ANOVA followed by one-way ANOVA within time points where interaction was observed. (**) denotes no interaction of time and treatment and a main effect of treatment \((p < 0.05)\). (‡) denotes no interaction of time and treatment and a main effect of both time and treatment \((p < 0.05)\). (*) denotes a significant effect of treatment for that time point \((p < 0.05)\). (†) denotes a significant main effect of dose from qRT-PCR dose-response analysis (Table 2.4). Values for time-matched vehicle controls are not shown.
Figure 2.5. Composition and expression patterns of significantly enriched GO categories from SAFE analysis. Panels A-D are SAFE plots for two commonly enriched categories for both deltamethrin (A & C) and permethrin (B & D). Panels E & F are SAFE plots for a category not enriched for either deltamethrin (E) or permethrin (F). The x-axis of each plot denotes the position of all probe set in a rank ordered list of significance (from left to right) according to the empirical $p$-value from a linear regression across dose. The y-axis is a cumulative percentage calculated by taking the rank position of a given probe set either within the entire data set (solid unity line) or the interrogated Gene Ontology sub-category (solid stair-step line) and dividing them by the total number of probe sets contained within the entire data set or interrogated category, respectively. The degree of deviation of the stair-step line from the unity line indicates enrichment. The probe sets (excluding ESTs) that are ranked highest in significance for each GO category for both compounds in panels A-B and C-D are denoted at the top of each panel and included in a heatmap to the side of the respective panels. In the heatmaps, each row of tiles is a probe set and each column of tiles represents the mean fold-change from control with increasing doses of each compound running from right to left. Colorbar for heatmaps is given in panel G.
Figure 2.6. Pyrethroid effects on branching and neurite length in primary cortical cell cultures. Changes in the total number neurite of branch points (A & D), total neurite length (B & E) and cell viability using an ATP-luciferase assay (C & F) in primary cortical cell cultures exposed to deltamethrin (A-C) or permethrin (D-F). n = 3 different cultures. Values for each end point are normalized to untreated controls (± standard error). Untreated control values are shown in white. The bold lines underneath each curve represent the range of estimated brain concentrations expected to occur during the in vivo exposures used in the present study (Mirfazaelian et al. 2006 and Tornero-Velez et al. 2007). Significance was determined using a One-way ANOVA, * = p < 0.10, ** = p < 0.05.
Works Cited


Pearson K. (1911) On the probability that two independent distributions of frequency are really samples from the same population. *Biometrika* **8**, 250-254.


Splice Variant Specific Induction of Ca^{2+}/calmodulin Dependent Protein Kinase 1-gamma mRNA in Response to Pyrethroid Exposure.

Chapter 3

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Abstract.

Pyrethroid insecticides induce neurotoxicity in mammals by interfering with ion channel function in excitable neuronal membranes. Previous work demonstrated dose-dependent increases in the expression of Ca$^{2+}$/calmodulin dependent protein kinase (Camk1g) mRNA following acute deltamethrin (DLT) and permethrin (PERM) exposure. The present study tests the hypothesis that changes in Camk1g expression in the rat following acute pyrethroid exposure are due to a specific increase in the Camk1g1 (Ca$^{2+}$-sensitive) splice variant and not the Ca$^{2+}$-insensitive splice variant Camk1g2. Long-Evans rats (n = 8 / group) were acutely exposed (p.o.) to PERM (1, 10, 40, 100 mg/kg), DLT (0.3, 1, 3 mg/kg) or corn oil vehicle. Frontal cortex was collected at 6 h post-dosing. In addition, rats were exposed to PERM (100 mg/kg) or DLT (3 mg/kg) and frontal cortex was collected at 1, 3, 6 or 9 hours along with time matched vehicle controls. Expression of Camk1g1 and Camk1g2 mRNA was measured by quantitative real-time RT-PCR and quantified with the $2^{-\Delta\Delta CT}$ method. Dose-dependent increases in Camk1g1 mRNA expression were observed for DLT and PERM at 6 h. In addition, a dose-dependent increase in Camk1g2 was also observed at 6 h with DLT only, although it was very small in magnitude (<1.2-fold). The increases in Camk1g1 expression for DLT and PERM peak between 3 & 6 h post-exposure and return to control levels by 9 h. There was no detectable increase in Camk1g1 protein as assessed with Western blots. This study demonstrates that following an acute in vivo exposure, deltamethrin and permethrin increase expression of Camk1g1 mRNA in rat cortex. These changes in Camk1g1 mRNA expression may be caused by the actions of pyrethroids at the neuronal membrane.
Introduction.

Pyrethroids are insecticides commonly used in a variety of agricultural, veterinary, domestic and human healthcare related applications (Heudorf and Angerer 2003). As the use of other insecticide classes such as organophosphates and organochlorines has declined, pyrethroid usage has risen. It is estimated that pyrethroids comprise ~25% of the world insecticide market (Amweg et al. 2003). Exposure assessments have detected pyrethroids and pyrethroid residues in the urine of pesticide applicators, blood samples of pregnant urban mothers, residential carpet dust, and in surface wipe samples from U.S. child-care centers (Leng et al. 1997; Whyatt et al. 2003; Colt et al. 2004; Weston et al. 2004; Tulve et al. 2006). These data indicate that the potential for human pyrethroid exposure is present in a variety of settings.

Pyrethroids are neurotoxicants that interact with voltage-sensitive ion channels expressed in nerve membranes (Narahashi 1996). The primary molecular targets of pyrethroids are voltage-sensitive Na\(^+\) channels (VSSCs) expressed in neuronal axons. Pyrethroids slow the deactivation of these channels and induce persistent Na\(^+\) currents that occur at membrane potentials when VSSCs are normally impermeable to Na\(^+\) (Na\(^+\) tail currents, see Narahashi 2001). Voltage sensitive Ca\(^{+2}\) channels and voltage-sensitive Cl\(^-\) channels have also been shown to be affected by pyrethroids (Burr and Ray 2004; Shafer and Meyer 2004; Symington and Clark 2005). The net effect of the pharmacological actions of pyrethroids at these channels is an alteration in neuronal firing patterns that is thought to underlie the poisoning symptoms and transient effects on behavior observed following acute \textit{in vivo} pyrethroid exposures (for review see Ray 2001; Soderlund et al. 2002; Wolansky and Harrill 2008). Alterations in neuronal firing patterns from pharmacological or sensory
stimuli have also been shown to trigger \textit{de novo} gene expression that contributes to long-lasting adaptive changes in neuronal form and function, such as late-phase long term potentiation and synaptic terminal remodeling (Bading 2000; West et al. 2002; Fields et al. 2005). To date, alterations in the expression of activity-regulated genes downstream of the pharmacological interactions of pyrethroids with molecular targets at the neuronal membrane remain poorly characterized.

Previous work in this laboratory on the acute \textit{in vivo} actions of pyrethroids revealed a dose-dependent increase in the expression of Ca^{2+}/calmodulin dependent protein kinase 1g (\textit{Camk1g}) mRNA in the frontal cortex of the rat following exposure to two pyrethroids: deltamethrin and permethrin (Harrill et al, \textit{submitted}). Treatment of \textit{in vitro} cultures of interconnected cortical neurons to pharmacological agents that increase spontaneous firing rates also results in an increase in \textit{Camk1g} mRNA expression (Xiang et al. 2007). Likewise, sub-chronic \textit{in vivo} exposure of laboratory animals to pharmacological agents known to increase spontaneous and sensory evoked neuronal firing patterns also results in an increase in \textit{Camk1g} mRNA expression (Lampl et al. 1998; Gronier and Rasmussen 1998; Dingledine 2005; Fatemi et al. 2006). Thus, the increase in \textit{Camk1g} mRNA expression following \textit{in vivo} pyrethroid exposure is consistent with increases in neuronal excitability elicited by these compounds.

In the rat brain, \textit{Camk1g} is expressed in two alternative mRNA splice variants that are transcribed from the same chromosomal locus (13q27). These splice variants are termed \textit{Camk1g1} and \textit{Camk1g2} and contain 13 and 12 exons, respectively (Nishimura et al. 2003). The domain structure of the protein encoded by the rat \textit{Camk1g1} splice variant includes a kinase catalytic domain, overlapping Ca^{2+}/calmodulin-binding and autoinhibitory domains,
C-terminal prenylation and palmitoylation motifs, and has 99% amino acid homology with the Camk1g1 protein expressed in mice (Nishimura et al. 2003; Takemoto-Kimura et al. 2003; Takemoto-Kimura et al. 2007). The activity of the Camk1g1 protein is dependent upon both the presence of Ca\(^{2+}\)-bound calmodulin and activation by an upstream CaM kinase kinase (Nishimura et al. 2003). In contrast, the rat Camk1g2 mRNA splice variant lacks exon 11 (see Figure 3.1). The transcribed Camk1g2 protein does not contain a Ca\(^{2+}/\)calmodulin-dependent binding domain and the kinase activity of this protein is not enhanced in the presence of Ca\(^{2+}\)-bound calmodulin (Nishimura et al. 2003). In addition, a frame shift in the amino-acid coding sequence of Camk1g2 occurs upon excision of exon 11, resulting in a C-terminal tail that lacks the lipidification sites present in Camk1g1 and essential to Camk1g1 function (Nishimura et al. 2003; Takemoto-Kimura et al. 2007). In previous studies that detected pyrethroid-induced increases in the expression of Camk1g mRNA in rat cortex, neither the Affymetrix microarray probe sets nor the qRT-PCR assays used distinguished between the expression of the Camk1g1 and Camk1g2 splice variants (Harrill et al. submitted).

Proteins in the Ca\(^{2+}/\)calmodulin dependent protein kinase family act as intracellular signaling molecules that detect transient increases in Ca\(^{2+}\) concentrations in the neuronal cytoplasm (such as those that occur in response to an incoming excitatory stimulus) and transduce those signals to downstream effector proteins by means of phosphorylation (Agell et al. 2002). In this manner activation of CaM-kinase cascades can bring about alterations in neuronal function in response to neuronal activity. A specific role of Camk1g in regulating activity-dependent outgrowth and branching in developing neurons has recently been described by Wayman et al. (2006) and Takemoto-Kimura et al. (2007). Both of these works
use constitutive overexpression or knock-down of Camk1g to demonstrate a role for Camk1g in controlling neuronal outgrowth in cultures derived from mouse hippocampus and rat cortex, respectively. In addition, these dendritogenesis studies used constructs based on the mouse and rat Camk1g1 protein. Wayman et al. (2006) demonstrate a Camk1g specific activation of the Ras/MEK/ERK signaling pathway in response to depolarization, a subsequent activation of the transcription factor CREB and transcription of Wnt-2, a soluble extracellular autocrine factor that promotes dendritic growth. Takemoto-Kimura et al. (2007) demonstrate that Camk1g is lipidified, inserted into membrane rafts that deliver the activated protein to dendritic arbors and controls neurotrophin stimulated dendrite growth and branching through the activation of a STEF/Rac signaling pathway. However, neither of these studies addressed the transcriptional regulation of Camk1g expression in response to changes in neuronal membrane excitability.

The highly divergent structure and functions of the proteins encoded by the two Camk1g splice variants, makes it important to determine whether pyrethroids alter expression of one or the other splice variants. Differential up-regulation of Camk1g1 or Camk1g2 mRNA by pyrethroids would have a different impacts on intraneuronal downstream signaling pathways because of the dissimilar structure and upstream regulatory mechanisms that control the kinase activity of these two proteins (Takemoto-Kimura et al. 2007). The goals of the present study included characterizing the effects of acute pyrethroid exposure on the expression of Camk1g1 and Camk1g2 mRNA splice variants in the rat frontal cortex, and determining if acute pyrethroid exposures in vivo result in a change in the expression of Camk1g1 protein. A low dose exposure paradigm was used (i.e. doses that ranged from below to slightly above the threshold for detecting changes in neurobehavioral function,
Wolansky et al. 2006) to prevent possible confounding effects of more highly toxic exposures.

**Methods.**

**Chemicals and dose solutions.** Permethrin (3-phenoxybenzyl (1R,S)-cis-trans-3-(2,2-dichlorovinyl)-2,2-dimethyl-cyclopropanecarboxylate, 92.0% purity, isomer composition: 40% cis, 60% trans, 1:1 ratio of 1R & 1S) and deltamethrin ((S)-cyano-(3-phenoxyphenyl)methyl (1R)-cis-3-(2,2-dibromovinyl)-2,2-dimethylcyclopropane carboxylate, 98.9% purity, isomer composition: 100% 1R, 3R, αS) were generously donated by FMC Corporation (Philadelphia, PA) and Bayer Cropscience (Research Triangle Park, NC), respectively. Chemical structures are shown in Figure 3.2. Pyrethroids were dissolved in corn oil (Sigma-Aldrich, St. Louis, MO) at 1, 10, 40 & 100 mg/mL permethrin and 0.3, 1 & 3 mg/mL deltamethrin. Dosing volume was 1mL/kg. qRT-PCR primers, probes and reagents were obtained from Applied Biosystems (Foster City, CA). AP7253b rabbit polyclonal antibody for Camk1g1 was obtained from Abgent, Inc. (San Deigo, CA). Mouse monoclonal antibody (sc-47751) for βII tubulin was obtained from Santa Cruz Biotechnologies, Inc. (Santa Cruz, CA). HRP-conjugated rabbit and mouse secondary antibodies were obtained from KPL, Inc. (Gaithersburg, MD).

**Animal care and treatment.** Male Long-Evans rats (49-62 days of age) were obtained from Charles River Laboratories (Wilmington, MA) and housed two per cage in standard polycarbonate hanging cages (45 cm X 24 cm X 20 cm) with heat sterilized pine shavings for bedding (Beta Chips, Northeastern Products, Inc., Warrensburg, NY). Animals were maintained on 12h:12h photoperiod (lighted hours: 06:00-18:00) and allowed a 5-7 day
period of acclimation to the colony prior to dosing. Colony rooms were maintained at 22.0 ± 2.0°C with a relative humidity of 55 ± 20%. Food (Purina 5001 Rat Chow) and tap water were provided *ad libitum*.

For the dose-response studies, rats were treated by oral gavage with 0.3, 1 or 3 mg/kg deltamethrin, 1, 10, 40 or 100 mg/kg permethrin or corn oil vehicle. Dose-effect data from Wolansky et al. (2006) were used to: 1) pick doses that were slightly below, at, and slightly above the threshold for detecting neurotoxic effects, and 2) assign equipotent dose-levels (EDL) to the administered doses to provide a comparative dose-metric between the two test compounds. Dosing for dose-response studies occurred between 06:30 and 07:00 hours and the last test subject was euthanized before 18:00 hours. Dosing and euthanasia times for individuals were counterbalanced across time of day. In the time course studies rats were treated via oral gavage with 3 mg/kg deltamethrin, 100 mg/kg permethrin or vehicle. All test subjects were dosed and euthanized between 07:30 & 17:30 hours. Rats were removed from the colony suite one hour prior to dosing and allowed to acclimate in a quiet holding room maintained under similar environmental conditions. Subjects were administered a single oral dose of test compound by gavage, and allowed to recover in their home cage prior to euthanasia for 6 hours (dose-response studies) or 1, 3, 6, 9, 12 or 24 hours (time course studies). Subjects were then individually removed to an adjoining suite with a separate HVAC system for euthanasia by decapitation. The facility was approved by the American Association for Accreditation of Laboratory Animal Care (AAALAC) and all experimental procedures were approved in advance by the US EPA, National Health and Environmental Effects Research Laboratory Animal Care and Use Committee.
Whole brains were rapidly removed and placed on a cold plate (4°C). The frontal cortex was removed by making a vertical incision at the anterior edge of the optic tract with a stainless steel razor, and rapidly frozen on a bed of dry ice. Cortical samples, without striatal tissue, were then bisected into contralateral hemispheres, weighed, frozen in liquid nitrogen and stored at -80°C. In the time course study, one contralateral hemisphere was used for RNA extraction and qRT-PCR assays while the other hemisphere was used to make protein homogenates for Western Blot analysis.

**RNA extraction.** Frontal cortex samples were homogenized in 1 mL of TRI Reagent (Molecular Research Center, Inc., Cincinnati, OH) per 50-100 mg of tissue using a Polytron® PT-K homogenizer (Kinematica, Lucerne, Switzerland) and total RNA was isolated per manufacturer’s instructions. Total RNA pellets suspended in DEPC-treated H₂O were then subject to DNase I treatment and reextracted with acid:phenol chloroform, pH = 4.7 and chloroform according to manufacturer’s protocol and resuspended in DEPC-treated H₂O until use (Ambion Inc., Austin, TX). The total RNA concentration of each sample was determined (absorbance @ 260 nm) on a Beckman-Coulter DU® 800 spectrophotometer (Fullerton, CA) and adjusted to 1.0 µg/µL prior to sample storage at -80°C. The ratio of absorbance values at 260 nm and 280 nm (Ab 260/280) was used to assess purity of total RNA samples. All samples used in these studies had Ab 260/280 ratios > 1.6 (data not shown). Preliminary PCR experiments using primers for rat β-actin genomic DNA (outlined in Tully et al. 2006) demonstrated that the above protocol adequately prevents genomic DNA contamination of total RNA samples (data not shown). In addition, the RNA integrity of each sample was determined using an Agilent 2100 Bioanalyzer and RNA 6000 Nano
LabChip Kit (Waldbron, Germany) according to manufacturer’s instructions. All samples used in qRT-PCR experiments had 18S:28S rRNA ratios > 1.6 (data not shown). Following the RNA purity and integrity screens, aliquots of each total RNA sample were stored at -80°C until use.

**Quantitative real-time RT-PCR.** qRT-PCR assays were performed using TaqMan® One-Step RT-PCR Master Mix Reagent Kits and TaqMan® Gene Expression Assays on a ABI 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA). The custom designed qRT-PCR assays used to distinguish between the *Camk1g1* and *Camk1g2* mRNA splice variants (Applied Biosystems, Foster City, CA) were targeted to exon junctions unique to the respective transcripts (see figure 3.1). AmpliTaq Gold® DNA Polymerase / dNTP mix, Multiscribe™ reverse transcriptase / RNA inhibitor mix and TaqMan® Gene expression primer-probe mix specific for the transcript of interest were combined according to manufacturer’s specifications (Applied Biosystems 2005). The reaction mixture was then dispensed into the reaction plate (15 µL / well) and 125 ng of total RNA (5 µL) was added. Each sample was measured in triplicate for each transcript of interest and internal reference gene. Reaction plates were maintained at 5°C during loading procedure. During data collection, reactions were incubated at 48°C for 45 min followed by incubation at 95°C for 10 min and 40 cycles of 94°C for 25 sec and 60°C for 1 min.

Details of the qRT-PCR assays used in this work are listed in Table 3.1. The amplification efficiency of each assay utilized in this experiment was examined using a serial dilution of pooled total RNA from rat cerebrocortex. Efficiencies were calculated as: $E_x = 10^{-1/m} - 1$, where $E$ is the amplification efficiency of target transcript $x$ and $m$ is the slope of
threshold cycles versus log_{10} [total RNA concentration] across the range of dilutions (Applied Biosystems 2004). All assays used in this experiment had amplification efficiencies in the range of 100 +/- 10% within the standard operating range of TaqMan assays suggested by the manufacturer (Applied Biosystems 2004). qRT-PCR data from deltamethrin and permethrin dose-response and time course studies were analyzed according to the 2^{\Delta\Delta C_T} method as described by (Livak and Schmittgen 2001). β-actin expression did not change as a function of time or dose for either compound (data not shown) and was used as the internal reference for all 2^{\Delta\Delta C_T} calculations. For dose-response studies, the mean ΔΔ^C_T of vehicle treated controls were used as the 2^{\Delta\Delta C_T} calibrator (Livak and Schmittgen 2001) to obtain approximations of fold-change from control. For time course studies, the mean ΔΔ^C_T of vehicle treated controls were used as the 2^{-\Delta\Delta C_T} calibrator for each time-matched treatment group.

Statistical analysis of qRT-PCR dose response data was performed using a two-way ANOVA with compound and equipotent dose level (EDL) as independent variables and 2^{-\Delta\Delta C_T} as the dependent variable followed by Dunnett’s mean contrast test. EDLs were defined as follows: group 1 (control) – vehicle controls for both compounds, group 2 (“sub-NOAEL”) – 0.3 mg/kg DLT & 1 mg/kg PERM, group 3 (“threshold”) – 1 mg/kg DLT & 10 mg/kg PERM, group 4 (“ED_{30}”) – 3 mg/kg DLT & 40 mg/kg PERM, group 5 (“ED_{50}”) – 100 mg/kg PERM. Statistical analysis of time course data was performed using a two-way ANOVA with time and treatment as independent variables and 2^{-\Delta\Delta C_T} as the dependent variable. Data were additionally analyzed with a one-way ANOVA at each time point with treatment as the independent variable (p < 0.05) to determine times of peak effect.
**Western blot analysis.** Cortical tissue was homogenized in a buffer containing (in mM):

- Tris-HCl (50), NaCl (150), EDTA (1), EGTA (1), Na$_3$VO$_4$ (1), 1 % Triton X-100, 1% C$_{24}$H$_{39}$NaO$_4$, 0.1 % SDS and 5 uL of Protease Inhibitor Cocktail III (Calbiochem, Inc., ) per 5 uL total buffer (pH = 7.4). Protein was homogenized with thirty even up and down strokes with a teflon pestle in a glass tube. Homogenates were then centrifuged at 12,000 x g at 4°C for 10 minutes. Supernatant was removed and protein concentrations measured using Pierce BCA™ Protein Assay Kit (Rockford, IL). Proteins were then stained with a 1:1 volume of Laemmli buffer (Bio-Rad, Hercules, CA) and stored at -80°C until use. On day of use, protein samples were boiled for 5 min and allowed to cool to room temperature. 12 µg of total protein for each sample was separated on 10% Precise™ pre-cast polyacrylamide gel (Pierce, Inc., Rockford, IL) with running buffer containing (in mM): Tris-HCl (100), HEPES (3), SDS (3), pH = 8.0. Gels were run at a constant voltage of 100 V for 15 min following by 125 V for 80 min. Proteins were then transferred to a nitrocellulose membrane using a TransBlot SD Semi-Dry Transfer cell (Bio-Rad, Inc., Hercules, CA) using a buffer containing (in mM): Tris-Base (48), Glycine (39), 0.000375% SDS and 20 % methanol. Proteins were transferred at 20 V for 30 min. Membranes were then washed with Tris-buffered saline (TBS, 42.1 mM Tris-HCl, 7.5 mM Tris-Base, 0.9% NaCl, pH=7.5) containing 0.03% Tween-20 and blocked for 1 h in 5% non-fat dry milk (Bio-Rad, Inc., Hercules, CA) diluted in TBS with 0.03% Tween-20. Membranes were then incubated overnight in blocking solution containing 0.03% Tween-20 and a 1:1000 dilution of *Camk1g1* primary antibody at 4°C. Membranes were then washed with TBS contained 0.01% Tween-20 and incubated with at 1:5000 dilution of rabbit HRP-conjugated secondary
antibody in blocking solution at room temperature for 1 h. Membranes were then washed again with TBS, 0.01% Tween-20 and incubated for 5 min at room temperature with SuperSignal® West Dura Extendend Duration Substrate (Pierce, Inc., Rockford, IL) according to manufacturer’s instructions. Blots were imaged (400 sec exposure) and optical densities of the stained bands quantified on a Bio-Rad VersaDoc™ Imaging system and Quantity One® 1-D Analysis software (v4.6, Bio-Rad, Inc., Hercules, CA). Membranes were then stripped for 10 min with Restore™ Western Blot Stripping Buffer (Bio-Rad). Membranes were sequentially reprobed with β-II tubulin primary antibody (1:2500) and mouse secondary antibody (1:10,000) and imaged according to the above protocol save that the primary incubation was for 1 h at room temperature and blots were visualized for 200 sec. Western blot expression ratios were calculated by normalizing each Camk1g signal to the corresponding β-tubulin signal and divided by the mean Camk1g/β-tubulin ratio from the control treated samples. Each time point was examined independently on separate gels.

Results.

Acute oral exposure of rats to 0.3 – 3 mg/kg deltamethrin or 1 – 100 mg/kg permethrin resulted in a dose-dependent increase in the expression of Camk1g1 in the frontal cortex at 6 h post-exposure (Figure 3.2C and 3.2D). The mRNA expression patterns observed using the Camk1g1 specific assay (red curve, Figure 3.2C and 3.2D) closely approximates, but does not exactly match, those observed using a qRT-PCR assay that detects both splice variants (blue curve, Figure 3.2A and 3.2B). For deltamethrin, expression of Camk1g1 was increased compared to control (~1.75-fold) across all doses at approximately the same level of expression. For permethrin, expression of Camk1g1 increased at 10 mg/kg and higher by ~2-fold greater compared to controls. This closely
matches the results of the Camk1g assay that detects both splice variants. A very small yet statistically significant increase in the expression of the Camk1g2 splice variant was observed at the ED$_{30}$ for motor activity (Wolansky et al. 2006) for each compound tested (Figure 3.2E and 3.2F). Results of the statistical analyses of these data are given in Table 3.2. It is apparent from these data that increased expression of the Camk1g1 splice variant is the main factor underlying the results obtained in the previous study by Harrill et al. (submitted).

The time course of Camk1g mRNA splice variant expression following either 3 mg/kg deltamethrin or 100 mg/kg permethrin exposures demonstrates that the increases in Camk1g1 and Camk1g2 are transient after an acute in vivo exposure (Figure 3.3A and 3.3B). Results of the statistical analyses of these data are given in Table 3.3. An appreciable increase in the expression of Camk1g1 mRNA is observed at 3 and 6 h with both compounds and returns to control levels by 9 h. The changes in Camk1g1 mRNA expression are larger in magnitude than those for Camk1g2 mRNA with both compounds. Similar to the dose-response data, the increases in Camk1g1 mRNA expression closely match the increases observed using the qRT-PCR assay that detects both splice variants. These data also support that increased expression of the Camk1g1 splice variant is the main contributor to the results obtained in the previous study (Harrill et al. submitted).

Since it was determined that Camk1g1 mRNA was changing most dramatically in response to pyrethroid exposure, alterations in Camk1g1 protein expression was examined in the in vivo time course samples used in this study. An antibody specific for a region the C-terminal tail of Camk1g1 (and not Camk1g2) was used to detect changes in protein levels. The predicted molecular weight of rat Camk1g1 protein is ~ 53 kDa. The Camk1g1 primary antibody detected a protein band with a slightly larger molecular weight (~ 60 kDa, Figure
Both antibodies were able to detect linear increases in expression of their respective proteins across a range of diluted protein standards (Figure 3.4C and D). β-tubulin was used as the loading control as treatment had no effect on expression (data not shown). No significant change in the expression of Camk1g1 protein was observed following treatment with either 3 mg/kg deltamethrin or 100 mg/kg permethrin (Figure 3.5).

Discussion.

The data from the present study demonstrate induction of Camk1g1 mRNA following acute in vivo exposure to both deltamethrin and permethrin. These exposures also resulted in modest and very low magnitude changes in the expression of the Camk1g2 mRNA splice variant. No changes in the expression of Camk1g1 protein was observed following acute exposure to either 3 mg/kg deltamethrin or 100 mg/kg permethrin, the highest doses used in this study. These data are consistent with increased Camk1g mRNA expression observed in the previous work using both Affymetrix microarray technology and a qRT-PCR assay capable of detecting both mRNA splice variants of this gene (Harrill et al., submitted). The present data expand on the previous finding by demonstrating that pyrethroid-induced changes in Camk1g are driven mainly by increased expression of the Camk1g1 splice variant. Furthermore, these data demonstrate that under the dosing conditions used in this study, increased Camk1g1 mRNA expression is transient, returning to control levels within 9 h, and does not lead to a concurrent increase in Camk1g1 protein expression.

It appears, at the least in the case of pyrethroid exposure, that expression of the rat Camk1g1 and Camk1g2 mRNA splice variants are not similarly regulated. Large treatment related effects on Camk1g2 mRNA expression are not present even when the expression of Camk1g1 mRNA is greatly affected. Currently, the intracellular mechanisms that control
inducible Camk1g1 or Camk1g2 mRNA expression are not known. Splice variant specific upregulation of activity-dependent mRNA species is not unprecedented in the central nervous system. Brain derived neurotrophic factor (Bdnf) mRNA be can expressed in a variety of splice variants in the CNS and different splice variants are expressed in response to different types, or different durations, of neuronal activity (Khundakar and Zetterstrom 2006; Liu et al. 2006). Differential activation of promoter elements upstream of the Bdnf untranslated exons controls the specific patterns of splice variant expression for this gene (Tabuchi et al. 2000). The promoter elements upstream of the Camk1g coding exons have not been examined. It is possible that differential activation of promoter elements in response to an excitatory stimulus may be the mechanism driving the differential expression observed here in response to pyrethroids. Alternatively, both Camk1g1 and Camk1g2 mRNA may be transcribed at the same rate even in the presence of pyrethroids. A pyrethroid-induced increase or decrease in the rate of Camk1g1 or Camk1g2 mRNA degradation, respectively, would also explain the differential response. Fukuchi et al. (2005) demonstrate that the half-life of mRNA expression for the pituitary adenylate cyclase-activating polypeptide (PACAP) increases in parallel to increases in the intensity of a depolarizing stimulus. Increased depolarization of neurons affected by pyrethroids may result in a stabilization of the expression of Camk1g1 mRNA and not Camk1g2, therefore resulting in divergent, stimulus-dependent expression patterns. The mechanism of differential Camk1g1 and Camk1g2 mRNA expression in the rat brain requires further investigation.

Changes in the expression of Camk1g mRNA are consistent with changes in neuronal excitability, such as those produced by pyrethroids (Vijverberg and Van den Bercken 1992; Song and Narahashi 1996; Narahashi 2001). Xiang et al. (2007) correlated firing rates of
cortical neuronal networks maintained in culture with global mRNA expression following
stimulation. They reported that patterns of *Camk1g* mRNA expression closely paralleled
increases in neuronal firing rates. Increased *Camk1g* mRNA expression (as well as the
increased neuronal firing rates) were dependent upon activation of voltage-sensitive sodium
channels (VSSC) and the presence of free Ca\(^{+2}\), as the VSSC blocker tetrodotoxin and the
Ca\(^{+2}\) chelator EGTA abolished both effects (Xiang et al., 2007). In separate studies, sub-
chronic dosing of olanzapine results in both increased burst firing in the frontal cortex *in vivo*,
and increased expression of *Camk1g* (Fatemi et al. 2005; Gronier and Rasmussen 2006). In
addition, *in vivo* exposure of rats to the antiepileptic drug phenytoin decreased *Camk1g*
mRNA expression (Dingledine et al. 2005). Phenytoin decreases neuronal firing rates in
several brain regions, including cortex (Matthews and Conner 1977; Lampl et al. 1998;
Calabresi et al. 1999). It is apparent that *Camk1g* mRNA expression is correlated with
alterations in neuronal firing rates. However, this previous research has not provided
information on which of the *Camk1g* splice variants is affected by the alterations in firing
rates. The present data suggest that in the rat the *Camk1g1* mRNA splice variant may be
more sensitive to changes in neuronal excitability given that the pyrethroids produce a more
robust increase in *Camk1g1* mRNA as compared to *Camk1g2* mRNA.

The finding that *Camk1g1* mRNA expression and not *Camk1g2* mRNA expression is
affected by pyrethroids is significant given that the biological activities of the proteins
encoded by these two splice variants are very distinct. *Camk1g1* kinase activity is dependent
upon the presence of Ca\(^{+2}\)-bound calmodulin and activation by an upstream CaM kinase
kinase whose own activity is also regulated by intracellular Ca\(^{+2}\) levels. *Camk1g2* kinase
activity is not enhanced in the presence of Ca\(^{+2}\)-bound calmodulin or affected by the
Camk1g1 upstream activating kinase (Nishimura et al. 2003). More importantly, the ability of Camk1g1 to regulate neuronal dendritogenesis in developing neurons is dependent upon sequential prenylation and palmitoylation of the C-terminal region of this protein (Takemoto-Kimura et al. 2007). Camk1g2 lacks the lipidification sites present on Camk1g1 required for mediating these effects. All studies reporting biologically significant effects of Camk1g on neuronal morphogenesis used the Camk1g1 splice variant as the basis for their study (Wayman et al. 2007; Takemoto-Kimura et al. 2007). No role for endogenous Camk1g2 protein has been identified. The induction of Camk1g1 mRNA in the present study indicates that pyrethroids may disrupt neuronal processes mediated by Camk1g1 protein such as dendritogenesis. Indeed, dendritic branching has been shown to be affected by both deltamethrin and permethrin in in vitro developmental neurotoxicity assays (Harrill et al. submitted). The induction of Camk1g1 mRNA observed here supports a putative role for the transcriptional induction of Camk1g1 in the pyrethroid-mediated effects on neurite branching.

The in vivo exposures utilized in the present time course study did not result in a significant increase in the expression of Camk1g1 protein. This is inconsistent with the increased expression of Camk1g1 mRNA. There are a number of possible explanations for this discrepancy. First, the Western blot assay used may not be detecting Camk1g1 protein. It is possible that a post-translational modification interferes with the ability of the Camk1g1 antibody to recognize the protein. The primary antibody used for quantification of Camk1g1 protein detected a strong protein band at ~ 60 kDa. This is larger than the predicted molecular weight of the rat Camk1g1 protein (53 kDa). The disparity between the predicted and observed Camk1g1 molecular weights is likely due to post-translational prenylation
and palmitoylation of the Camk1g1 protein (Takemoto-Kimura et al. 2007). This suggests that the 60 kDa band may be modified Camk1g1. Alternatively, this band may represent a non-specific recognition of a different protein by the primary antibody. Additional studies with purified Camk1g1 protein may help resolve this issue.

In contrast, the lack of measurable increases in Camk1g1 protein could be due to the acute dosing paradigm used in the present research. As demonstrated in Figure 3.3, the pyrethroid-induced increases in Camk1g1 mRNA expression are transient and recover on a time scale that reflects the pharmacokinetics of these compounds. Acute oral exposure of rats to permethrin and deltamethrin results in a rapid accumulation of these compounds in the brain. Peak concentrations occur between ~1-3 h and these compounds are cleared from the brain with half-lives on the order of tens of hours (Anadon et al. 1991; Anadon et al. 1996; Mirfazaelian et al. 2006; Tornero-Velez et al. 2007). Triggering an appreciable increase in Camk1g1 protein in adult neurons may require a more sustained increase in Camk1g1 mRNA then what is observed in the present study. More persistent (i.e., sub-chronic) exposures to pyrethroids that produce a steady-state brain burden of these compounds may be necessary to elevate Camk1g1 protein, but this is yet to be determined.

In summary, this study demonstrates that following an acute in vivo exposure to either deltamethrin or permethrin transiently increased expression Camk1g1 splice variant mRNA in the rat frontal cortex. The transient increases in Camk1g1 mRNA did not result in measureable increases in Camk1g1 protein under the exposure conditions used here. Future research efforts concerning Camk1g, neuronal excitability and pyrethroids should focus on determining how pyrethroid actions at the neuronal membrane results in the increased
expression of \textit{Camk1g1} mRNA and whether this response impacts neuronal structure or function.

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### Table 3.1. Camk1g and Camk1g splice variant specific TaqMan assays.

<table>
<thead>
<tr>
<th>Assay</th>
<th>GenBank Accession</th>
<th>Nucleotide Sequences</th>
<th>Nucleotide Alignments</th>
<th>Exon Junction</th>
<th>Amplification Efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Camk1g</td>
<td>NM_182842 Reference&lt;sup&gt;a,b&lt;/sup&gt;: 5'-GATGACATTTCTGAGTCAGCCAAGG-3' 810 → 834 8-9 94.2 %</td>
<td>810 → 834 8-9</td>
<td>810 → 834 8-9</td>
<td>96.0 %</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AB101231</td>
<td>810 → 834 8-9</td>
<td>810 → 834 8-9</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>AB101232</td>
<td>810 → 834 8-9</td>
<td>810 → 834 8-9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Camk1g1</td>
<td>NM_182842 Forward: 5'-CCTCCAGATTCAAGAAGACTTTGC-3' 956 → 979 10-11 94.2 %</td>
<td>956 → 979 10-11</td>
<td>1017 ← 1004 10-11</td>
<td>94.2 %</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AB101231 Backward: 5'-CGGCGGGCCGGCATTTG-3'</td>
<td>Backward: 5'-CGGCGGGCCGGCATTTG-3'</td>
<td>Backward: 5'-CGGCGGGCCGGCATTTG-3'</td>
<td>Backward: 5'-CGGCGGGCCGGCATTTG-3'</td>
<td>Backward: 5'-CGGCGGGCCGGCATTTG-3'</td>
</tr>
<tr>
<td></td>
<td>AB101232 Reporter: 5'-AAGTGGAGGGAACTTCAA-3'</td>
<td>Reporter: 5'-AAGTGGAGGGAACTTCAA-3'</td>
<td>Reporter: 5'-AAGTGGAGGGAACTTCAA-3'</td>
<td>Reporter: 5'-AAGTGGAGGGAACTTCAA-3'</td>
<td>Reporter: 5'-AAGTGGAGGGAACTTCAA-3'</td>
</tr>
</tbody>
</table>

<sup>a</sup>Primer and probe sequences of Applied Biosystems pre-designed TaqMan® qRT-PCR assays are proprietary. Reference sequence provided by manufacturer is listed.

<sup>b</sup>TaqMan® gene expression assay Rn00788224_m1.
**Table 3.2. Statistical analysis of qRT-PCR dose-response data.**

<table>
<thead>
<tr>
<th>Gene</th>
<th>EDL</th>
<th>COMPOUND</th>
<th>EDL*COMPOUND</th>
</tr>
</thead>
<tbody>
<tr>
<td>Camk1g</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EDL</td>
<td>F (4, 54) = 4.47, ( p = 0.0034^{**} )</td>
<td>3, 4, 5(^b)</td>
<td></td>
</tr>
<tr>
<td>COMPOUND</td>
<td>F (1, 54) = 0.96, ( p = 0.3370 )</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EDL*COMPOUND</td>
<td>F (3, 54) = 1.16, ( p = 0.3335 )</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Camk1g1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EDL</td>
<td>F (4, 54) = 2.79, ( p = 0.0355^{**} )</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>COMPOUND</td>
<td>F (1, 54) = 0.04, ( p = 0.8380 )</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EDL*COMPOUND</td>
<td>F (3, 54) = 0.78, ( p = 0.5096 )</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Camk1g2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EDL</td>
<td>F (4, 54) = 2.57, ( p = 0.0484^{**} )</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>COMPOUND</td>
<td>F (1, 54) = 6.76, ( p = 0.0120^{**} )</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EDL*COMPOUND</td>
<td>F (3, 54) = 1.95, ( p = 0.1325 )</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\)Data were analyzed using a two-way ANOVA model with equipotent dose level (EDL) and COMPOUND (deltamethrin, permethrin) as the independent variables. \(^b\)A post-hoc Dunnett’s many-to-one mean contrast test was also performed on the data. Numbers represent the EDL with means in expression significantly different from vehicle controls (\( p < 0.05 \) significance level), 3 = NOAEL, 4 = ED\(_{30}\) and 5 = ED\(_{50}\) for decreases in ambulatory motor activity. ** = significant at \( p < 0.05 \) in two-way ANOVA model. Analysis were performed on 2\(-\Delta_{\Delta}^{C}T\) values (Livak and Schmittgen 2001)
Table 3.3. Statistical analysis of qRT-PCR time course data.a

<table>
<thead>
<tr>
<th>Gene</th>
<th>TRT</th>
<th>F (1, 56) =</th>
<th>p =</th>
<th>h</th>
<th>TRT</th>
<th>F (1, 56) =</th>
<th>p =</th>
<th>n.e.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Camk1g</td>
<td></td>
<td>4.56</td>
<td>0.0371**</td>
<td>3 h</td>
<td></td>
<td>2.16</td>
<td>0.1472 n.e.</td>
<td></td>
</tr>
<tr>
<td>TIME</td>
<td>F (3, 56) = 1.75, p = 0.1665</td>
<td>TIME</td>
<td>F (3, 56) = 1.14, p = 0.3391</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TRT*TIME</td>
<td>F (3, 56) = 2.07, p = 0.1140</td>
<td>TRT*TIME</td>
<td>F (3, 56) = 0.54, p = 0.6586</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Camk1g1</td>
<td></td>
<td>4.76</td>
<td>0.0334**</td>
<td>3 h</td>
<td></td>
<td>4.65</td>
<td>0.0354** n.e.</td>
<td></td>
</tr>
<tr>
<td>TIME</td>
<td>F (3, 56) = 3.67, p = 0.0174**</td>
<td>TIME</td>
<td>F (3, 56) = 0.76, p = 0.5225</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TRT*TIME</td>
<td>F (3, 56) = 3.92, p = 0.0131**</td>
<td>TRT*TIME</td>
<td>F (3, 56) = 0.37, p = 0.7784</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Camk1g2</td>
<td></td>
<td>6.13</td>
<td>0.0164**</td>
<td>6 h</td>
<td></td>
<td>6.89</td>
<td>0.0112** 6 h</td>
<td></td>
</tr>
<tr>
<td>TIME</td>
<td>F (3, 56) = 5.25, p = 0.0029**</td>
<td>TIME</td>
<td>F (3, 56) = 4.73, p = 0.0052**</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TRT*TIME</td>
<td>F (3, 56) = 4.92, p = 0.0042**</td>
<td>TRT*TIME</td>
<td>F (3, 56) = 4.47, p = 0.0069**</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

aData were analyzed using a Two-way ANOVA model with treatment (TRT) and TIME as the independent factors. ** = significant at p < 0.05. bData were additionally analyzed within time point for each gene using a One-way ANOVA. Times noted on this table were significant at p < 0.05.
Figure 3.1. Exon schematic of rat Camk1g1 and Camk1g2. A) Intron/exon diagram of rat Camk1g1 and Camk1g2. Boxes are exons while lines are introns. Camk1g1 contains 13 exons while Camk1g2 contains 12 exons, with the calmodulin-binding domain of Camk1g1 (exon 11) absent. Highlighted boxes correspond to the exon junctions targeted by the qRT-PCR assays. B) Assay schematics for Camk1g, Camk1g1 and Camk1g2 assays, respectively. Each assay contains a forward primer, reverse primer and a probe sequence with fluorophore (P) and quencher (Q). Upon primer extension the 5’ to 3’ nuclease activity of the AmpliTaq Gold DNA polymerase degrades the primer, releases the fluorophore from the quencher and light is emitted which is quantified for calculations of mRNA abundance. Note the probe and reverse primer of the Camk1g1 and Camk1g2 assays are unique while the forward primer of each of these two is the same. Assays are described in detail in Table 3.1.
Figure 3.2. Dose-response for Camk1g1 and Camk1g2 mRNA expression. Panels A-F show dose-response curves for the Camk1g (A-B), Camk1g1 (C-D) and Camk1g2 (E-F) qRT-PCR assays in frontal cortex at 6 h following acute exposure to deltamethrin (A,C & E) or permethrin (B,D & F). Data were quantified and analyzed using the $2^{-\Delta\Delta CT}$ method and data are expressed as mean $2^{-\Delta\Delta CT}$ ± standard error. Numbers underlying the points in panels A & B demonstrate which equipotent dose level (EDL) groupings each treatment belongs to for statistical analyses of these data: 1 = vehicle control, 2 = sub-NOAEL, 3 = threshold, 4 = ED$_{30}$, 5 = ED$_{50}$ for decreases in motor activity. Results of statistical analyses of these data are given in Table 3.2. * = treatment group mean is significantly different from vehicle control in a Dunnett’s many-to-one mean contrast test (p < 0.05) ** = main effect of equipotent dose level with no interaction of EDL & compound in Two way ANOVA (p < 0.05). *** = significant interaction of EDL & COMPOUND.
Figure 3.3. Time course for Camk1g1 and Camk1g2 mRNA expression. Rats were orally exposed to either 3 mg/kg deltamethrin (A) or 100 mg/kg permethrin (B) and mRNA expression quantified in the frontal cortex at 1, 3, 6 and 9 h post-exposure. Data within each time point were quantified using the $2^{-\Delta\Delta CT}$ method and data are expressed as mean $2^{-\Delta\Delta CT} \pm$ standard error. Red bars correspond to the Camk1g1 specific qRT-PCR assay, green bars to the Camk1g2 specific qRT-PCR assay and blue bars to the assay that detects both splice variants. Results of statistical analyses of these data is given in Table 3.3. * = main effect of treatment with no interaction of time & treatment in a two-way ANOVA ($p < 0.05$). ** = significant interaction of time & treatment ($p < 0.05$). Arrows denote time points with means in expression significantly different from time-matched vehicle control values in a One-Way ANOVA ($p < 0.05$). Vehicle treated control data not shown.
Figure 3.4. *Rat Camk1g1 and Camk1g2 protein sequences and Camk1g1 protein assay development.* A) Alignment of rat *Camk1g1* and *Camk1g2* protein coding sequences. *Camk1g1* is the top line and *Camk1g2* the bottom line in each doublet. Highlighting colors correspond to the strength of identity between the matched amino acids with red being absolute identity and blue being a poor match. Color bar is in top right corner. Protein alignment performed using Swiss Institute for Bioinformatics TCoffee tool. The antigen sequence for the *Camk1g1* primary antibody is marked with a blue box. B) A single strong band at ~60 kDa was detected using the *Camk1g1* primary antibody. C) Signal from *Camk1g1* increases linearly as a function of total protein loaded from 5-20 µg. Visualization of Western blot bands given in top right corner of panel. D) Signal from β-tubulin primary antibody increased linearly as a function of total protein loaded from 5-20 µg after stripping of *Camk1g1* primary antibody. Standard curves in C and E were obtained from the same blot.
Figure 3.5. Camk1g1 protein expression following acute pyrethroid exposure. Rats were orally exposed to either 3 mg/kg deltamethrin (A) or 100 mg/kg permethrin (B) and Camk1g1 protein expression was quantified in frontal cortex via Western blots at 1, 3, 6, 9, 12 or 24 h post-exposure. Signal intensities for Camk1g1 expression were normalized within sample to β-tubulin and expressed as percent change from untreated control within each set of treated and time-matched control samples. Data are expressed as mean fold-change from time matched, vehicle-treated control groups ± S.E.
Works Cited


Takemoto-Kimura, S., Terai, H., Takamoto, M., Ohmae, S., Kikumura, S., Segi, E.,
and characterization of CLICK-III/CaMKIgamma, a novel membrane-anchored


Transcriptional Response of Rat Frontal Cortex to Acute Pyrethroid Exposure.

Chapter 4

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Abstract.

Pyrethroids are pesticides that interact with voltage-sensitive ion channels and disrupt neuronal function. Acute disruption of neuronal function can result in adverse effects on behavior. In addition, change in neuronal firing patterns, such as those produced by pyrethroids, may also lead to changes in activity-regulated gene expression. Previous research demonstrated that permethrin (a Type I pyrethroid) and deltamethrin (a Type II pyrethroid) exposure alters gene expression in the rat cortex. The present study expands upon the previous findings by examining the global transcriptional response of rat cortex for six different pyrethroids. Rats were acutely dosed with a Type I pyrethroid (permethrin, bifenthrin or tefluthrin), a Type II pyrethroid (deltamethrin, cypermethrin or cyfluthrin) or vehicle and cortex was sampled at 3 and 6 h post exposure. Affymetrix GeneChips® were used to obtain global gene expression profiles from the cortical tissue. Analysis of the microarray data demonstrated robust transcriptional responses following exposure to Type II pyrethroids. Type I pyrethroids produced only marginal effects on cortical gene transcription some of which were in common with the effect produced by the Type II pyrethroids. Comparison of gene expression profiles revealed a suite of genes commonly affected by the Type II pyrethroids. Ingenuity® pathway analysis of these transcriptional changes yielded an interconnecting network of gene interactions that is consistent with the excitatory effects of these compounds.
Introduction.

Pyrethroids are neurotoxic pesticides used in a variety of agricultural, household, veterinary and human health applications (Heudorf & Angerer 2001; Yanez et al. 2002). Use of pyrethroids has increased in conjunction with decreased use of insecticides from other chemical classes. There is a potential for human exposures as pyrethroids and pyrethroid residues have been detected in urine samples from pesticide applicators, blood samples of urban mothers, residential house dust and wipe samples from U.S. child care centers (Leng et al. 1997; Whyatt et al. 2003; Colt et al 2004; Weston et al. 2004; Tulve et al. 2006).

Classically, pyrethroids have been divided into different sub-types based chemical structure and the signs of acute poisoning observed in laboratory rodents at near lethal dose levels (Verschoyle and Aldridge 1980; Lawrence and Casida 1982). Pyrethroids containing an α-cyano group (Type II) produce a poisoning syndrome characterized by increased burrowing and digging behavior that progresses to whole body tremor followed by profuse salivation and a sinuous writhing known as choreoathetosis (CS-syndrome, Verschoyle and Aldridge 1980). Pyrethroids that lack an α-cyano group (Type I) produce a poisoning syndrome characterized by episodes of aggressive sparing behavior and increased sensitivity to external stimuli that progresses to fine tremors followed by course whole body tremors and prostration (T-syndrome, Verschoyle and Aldridge 1980). At lower dose levels, the neurotoxic effects of pyrethroids have been detected by a number of neurobehavioral tests. Some behavioral endpoints are commonly affected by all pyrethroids (i.e. motor activity, Wolansky et al. 2006), while others are affected differently across the pyrethroid class (e.g. acoustic startle response and thermoregulation, Crofton and Reiter 1988; Wolansky and Harrill 2007).
The primary molecular targets of pyrethroids are voltage-sensitive sodium channels (VSSCs). Pyrethroids delay VSSC deactivation which results in a prolonged entry of Na\(^+\) into the neuron (Na\(^+\) tail current) during periods when unmodified VSSCs are normally impermeable (Narahashi 2001). Abnormal Na\(^+\) tail currents can persist for as little as milliseconds and as long as hundreds of milliseconds depending upon the structure of the pyrethroid and the isoform of VSSC (Vijverberg and Van den Bercken 1990; Smith and Soderlund 1998; Choi and Soderlund 2006). Type II pyrethroids consistently produce more prolonged Na\(^+\) tail currents than Type I pyrethroids (Vijverberg and Van den Bercken 1990; Song et al. 1996; Choi and Soderlund 2006). The result of increased Na\(^+\) permeability through VSSCs is repetitive neuronal action potential firing following a stimulus and an overall change in neuronal firing rates (Vijverberg and Van den Bercken 1990; Song and Narahashi 1996). Some pyrethroids also affect a number of secondary molecular targets such as voltage-sensitive Ca\(^{2+}\) channels, voltage-sensitive Cl\(^-\) channels (Burr and Ray 2004; Shafer and Meyer 2004; Symington and Clark 2007). Activation of these channels may or may not contribute to the manifestation of acute neurotoxicity \textit{in vivo} or the difference in the acute signs of poisoning observed with the two pyrethroid types (Soderlund et al. 2002; Ray and Fry 2006).

The accepted mode-of-action for the acute adverse effects of pyrethroids includes disruption of voltage-sensitive ion channel function and subsequent increases in neuronal excitability which, in turn, leads to behavioral effects observed at the whole organism level (Ecobichon 2001; Soderlund et al. 2002; Ray and Fry 2006). Changes in neuronal excitability also triggers changes in gene expression that may result in alterations in neuronal function (Fields et al. 1997; Finkbeiner and Greenberg 1998; Fields et al. 2005; Tropea et al.
To date, the effects of acute pyrethroid exposure on activity-dependent gene expression in the nervous system has not been extensively studied. Questions remain regarding which genes are altered following pyrethroid exposure, whether or not the same sets of genes are affected by different compounds within the pyrethroid class and what intracellular signal transduction mechanisms are activated to trigger pyrethroid-induced changes in gene expression.

Previous work using oligonucleotide microarrays demonstrated that acute pyrethroid exposure, at doses near the threshold for producing behavioral effects in the rat, altered gene expression in the frontal cortex (Harrill et al. submitted). The gene expression changes observed in that study were consistent with pyrethroid-induced increases in neuronal excitability in vivo. In addition, this study demonstrated both differences and similarities in the overall transcriptional response elicited by permethrin and deltamethrin, the two pyrethroids tested. The present study expands upon these findings by examining global gene expression patterns in the rat frontal cortex across time following acute exposure to six pyrethroids: permethrin, bifenthrin and tefluthrin (Type I) or deltamethrin, cypermethrin or cyfluthrin (Type II). Equipotent dose levels were selected based on the behavioral data of Wolansky et al. (2006). Global gene expression profiles from Affymetrix GeneChips® were compared across compounds and functional gene regulatory networks were constructed from commonly altered genes.

Methods.

Chemicals. All pyrethroids examined in this study were of technical grade. Bifenthrin (BIF: 98.9 % purity), permethrin (PERM: 92 % purity) and cypermethrin (CYP: 88.0 % purity) were donated by FMC Corporation (Philadelphia, PA). Deltamethrin (DLT: 98.9 % purity)
and β-cyfluthrin (CYF: 99.2 % purity) were donated by Bayer Cropscience (Research Triangle Park, NC). Tefluthrin (TEF: 92.6 % purity) was donated by Syngenta Crop Protection (Greensboro, NC). Chemical structures for the six pyrethroids are provided in Figure 4.1. Complete IUPAC nomenclature and isomer ratios of the test material are available in Wolansky et al. (2006). Equipotent dose levels (ED$_{30}$ for decreased motor activity) for each of the pyrethroids were selected based on data from Wolansky et al. (2006) (see Figure 4.2). Pyrethroids were dissolved in corn oil (Sigma-Aldrich, St. Louis, MO) with a dosing volume of 1 mL/kg.

**Animal care and treatment.** Male Long-Evans rats (58 days of age) were obtained from Charles River Laboratories (Wilmington, MA) and housed two per cage in standard polycarbonate hanging cages (45 cm X 24 cm X 20 cm) with heat sterilized pine shavings for bedding (Beta Chips, Northeastern Products, Inc., Warrensburg, NY). Animals were maintained on 12h:12h photoperiod (lighted hours: 06:00-18:00) and allowed a 5 to 7 day period of acclimation to the colony prior to dosing. Colony rooms were maintained at 22.0 ± 2.0°C with a relative humidity of 55 ± 20%. Food (Purina 5001 Rat Chow) and tap water were provided *ad libitum*. The facility was approved by the American Association for Accreditation of Laboratory Animal Care (AAALAC) and all experimental procedures were approved in advance by the US EPA, National Health and Environmental Effects Research Laboratory Animal Care and Use Committee.

Rats received a single, oral dose of either 43 mg/kg PERM, 10.7 mg/kg CYP, 3.2 mg/kg BIF, 3 mg/kg DLT, 2.3 mg/kg TEF, 2.2 mg/kg CYF or corn oil vehicle and allowed to recover for either 3 or 6 h. These doses are an approximate ED$_{30}$ for decreased motor
activity based on the data of Wolansky et al. (2006). Rats were treated and tissues collected in four separate blocks in this study. Blocks 1 and 2 contained rats treated with Type I pyrethroids (PERM, BIF, TEF) or Type II pyrethroids (DLT, CYP, CYF) for 3 h (n = 3 / block, n = 6 / treatment). Blocks 3 and 4 contained rats treated with Type I pyrethroids or Type II pyrethroids for 6 h (n = 3 / block, n = 6 / treatment). Each block also contained time matched vehicle controls (n = 3 / block). Dosing and euthanasia times for pyrethroid and vehicle treated rats was counterbalanced across time of day within each block. All dosing occurred between 09:00 and 12:00 hours and the last test subject was euthanized before 18:00 hours. Rats were removed from the colony suite 1 h prior to dosing and allowed to acclimate in a quiet holding room maintained under similar environmental conditions. Subjects were administered a single oral dose of test compounds by gavage and allowed to recover in their home cage prior to tissue sampling. Subjects were then individually removed to an adjoining suite with a separate HVAC system for euthanasia by decapitation.

Whole brains were rapidly removed and placed on a cold plate (4°C). The frontal cortex was dissected by making a vertical incision at the anterior edge of the optic tract with a stainless steel razor, and rapidly frozen on a bed of dry ice. Cortical samples, without striatal tissue, were then bisected into contralateral hemispheres, weighed, frozen in liquid nitrogen and stored at -80°C.

**RNA extraction.** Cortical samples were homogenized in 1 mL of TRI Reagent (Molecular Research Center, Inc., Cincinnati, OH) per 50-100 mg of tissue using a Polytron® PT-K homogenizer (Kinematica, Lucerne, Switzerland) and total RNA was isolated per manufacturer’s instructions. Total RNA pellets suspended in DEPC-treated H₂O were then
subject to DNase I treatment and re-extracted with acid:phenol chloroform, pH = 4.7 (Ambion Inc., Austin, TX) and chloroform according to manufacturer’s protocol and resuspended in DEPC-treated H₂O until use. The total RNA concentration of each sample was determined (absorbance @ 260 nm) on a Beckman-Coulter DU® 800 spectrophotometer (Fullerton, CA) and adjusted to 1.0 µg/µL prior to sample storage at -80°C. The ratio of absorbance values at 260 nm and 280 nm (Ab 260/280) was used to assess purity of total RNA samples. All samples used in these studies had Ab 260/280 ratios > 1.83 (data not shown). Preliminary PCR experiments using primers for rat β-actin genomic DNA (outlined in Tully et al. 2006) demonstrated that the above protocol adequately prevents genomic DNA contamination of total RNA samples (data not shown). In addition, the RNA integrity of each sample was determined using an Agilent 2100 Bioanalyzer and RNA 6000 Series II Nano LabChip Kit (Waldbron, Germany) according to manufacturer’s instructions. All samples used in microarray and qRT-PCR experiments had 18S:28S rRNA ratios > 1.8 and RNA Integrity Numbers > 8.8 (data not shown). Following the RNA purity and integrity screens, aliquots of each total RNA sample (1 µg/µL for microarray hybridization or 25 ng/µL for qRT-PCR assays) were stored at -80°C until use.

**Microarray sample preparation and data collection.** Affymetrix Rat Genome 230 2.0 GeneChip® oligonucleotide microarrays (Santa Clara, CA) were used in this experiment. First and second strand cDNA synthesis, cRNA amplification, clean-up and biotin-labeling of each sample were performed with BioArray™ single-round RNA amplification and biotin labeling system (Enzo Life Sciences, Farmingdale, NY) and Qiagen RNeasy spin columns (Spoorstraat, Netherlands), respectively, according to manufacturer’s instructions. Biotin-
labeled cRNA was fragmented using Affymetrix 5X fragmentation buffer (200mM Tris acetate - pH=8.1, 100 mM KOAc, 150 mM MgOAc).

Fragmentation of biotin-labeled cRNA, GeneChip® hybridizations, washes and staining were performed according to standard Affymetrix protocols (Affymetrix 2004). Hybridizations were performed in an Affymetrix Hybridization Oven 640. Washes were performed on an Affymetrix Fluidics Station 450 using the EukGE-WS2v4-450 fluidics script. GeneChips® were scanned using an Affymetrix GeneChip® 3000 Scanner with the GCOS v1.4.0.036 software package. Target intensity was set to a value of 500 with all other scanning parameters set at the factory defaults. The 3`/5` ratios for GAPDH and β-actin internal controls genes were within the range suggested by the manufacturer, indicating that RNA was not degraded during processing. The intensity of hybridization controls (BioB, BioC, BioD and Cre) increased linearly on all arrays.

**Microarray data analysis.** Gene expression profiles for all samples in the study were collapsed across block for data analysis. Gene expression summaries were calculated with RMAExpress© v4.7 (University of California at Berkeley) using the Robust Multiarray Average method (RMA, Irizarry et al. 2003). Paired t-tests between treated samples and time-matched controls were performed using Microsoft Excel® to obtain an initial global overview of the data. Treatment-related changes in gene expression were detected using the Linear Models for Microarray Data (LIMMA, Smyth 2005) package in the R statistical computing environment (v2.6.1). Each compound was analyzed separately in conjunction with time-matched vehicle control values according to contrast matrix outlined in Figure 4.4A. The comparisons made for each probe set using LIMMA followed the general model
for a two-way analysis of variance (ANOVA) with time and treatment as the independent factors (Figure 4.4B). Treatment-related changes in expression were determined according to the flowchart in Figure 4.4C. Main effects of time and treatment and the interaction between time and treatment were considered significant at a false discovery rate adjusted \( p \)-value < 0.10.

The Significant Analysis of Function and Expression (SAFE, Barry et al. 2005) method was used to identify pathways and functional categories whose genes are coordinately regulated in a treatment-related fashion. SAFE and array annotation were loaded from Bioconductor (v2.1, Gentleman et al. 2004). A comparative SAFE analysis was also used to test for enrichment of gene sets with treatment related changes in expression for each of the individual pyrethroids (according to LIMMA) across all the other pyrethroids in the test panel. Prior to implementation of the SAFE analyses data were normalized (or re-centered) to the combined mean of the vehicle treated control groups collapsed across time. This normalization was necessary due to the inability of the SAFE method to simultaneously analyze two independent variables. Normalization removed the main effects of time from the data (Appendix B, Figure 1). A multi-factor one-way ANOVA \( F \)-statistic was used as the local statistic in the SAFE analyses to assess treatment related change in expression within the four treatment groups for each pyrethroid. The global statistic for enrichment was a Wilcoxon ranked sum test (Ott 2003). 1000 permutations of the treatment groups were used by SAFE to assess the significance of the entire procedure using the Yekutieli and Benjamini (1999) method to control the false discovery rate (FDR) and account for multiple comparisons. Ingenuity® pathway analysis software (Ingenuity Inc., Redwood City, CA)
was also used to construct interactive gene networks from probe sets altered by pyrethroid treatment.

**Quantitative real-time RT-PCR.** qRT-PCR was performed using TaqMan® One-Step RT-PCR Master Mix Reagent Kits and TaqMan® Gene Expression Assays on a ABI 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA). AmpliTaq Gold® DNA Polymerase / dNTP mix, Multiscribe™ reverse transcriptase / RNA inhibitor mix and TaqMan® Gene expression primer-probe mix specific for the transcript of interest were combined according to manufacturer’s specifications (Applied Biosystems, 2005). The reaction mixture was then dispensed into the reaction plate (15 µL / well) and 125 ng of total RNA (5 µL) was added. Each sample was measured in triplicate for each transcript of interest and internal reference gene. Reaction plates were maintained at 5°C during loading procedure. During data collection, reactions were incubated at 48°C for 45 min followed by incubation at 95°C for 10 min and 40 cycles of 94°C for 25 sec and 60°C for 1 min.

qRT-PCR assays were designed via the Applied Biosystems (ABI) primer / probe selection algorithm and bioinformatics pipeline (Applied Biosystems 2006). The amplification efficiency of each assay was examined using a serial dilution of pooled total RNA from rat cortex. Efficiencies were calculated as: $E_x = 10^{(-1/m)} - 1$, where E is the amplification efficiency of target transcript x and m is the slope of threshold cycles vs log [total RNA concentration] across the range of dilutions (Applied Biosystems 2004). Assay identification numbers, context sequences, amplicon lengths and calculated amplification efficiencies are listed in Appendix B, Table 1.
Six transcripts identified as having treatment related changes in expression were examined by qRT-PCR to confirm results of the microarray data. qRT-PCR data was analyzed according to the $2^{\Delta\Delta C_T}$ method as described by (Livak and Schmittgen 2001). β-actin expression did not change as a function of time or treatment for either compound (data not shown) and was used at the internal reference for all $2^{\Delta\Delta C_T}$ calculations.

The mean ΔΔC_T of vehicle treated controls within each time was used as the $2^{\Delta\Delta C_T}$ calibrator for each time-matched treatment group. A two-way ANOVA was used to statistically analyze the qRT-PCR data with time and treatment as independent variables and $2^{\Delta\Delta C_T}$ as the dependent variable ($p < 0.05$ for each contrast). Transcripts with a significant time*treatment interaction were additionally analyzed with a one-way ANOVA at each time point with treatment as the independent variable ($p < 0.05$).

**Results.**

The type II pyrethroids had a much more dramatic effect on gene expression in the frontal cortex than Type I pyrethroids. Figure 4.3 plots the magnitude of fold change from control versus the empirical $p$-value for pair-wise comparisons of treatment and control samples for each pyrethroid tested at each time point in the study. Note that the statistical test used in this figure is not adjusted for multiple comparisons or low abundance transcripts and was not used in the present study as final indication of significance. Instead, these comparisons were used to examine global trends in the gene expression data. For all the pyrethroids tested, at both 3 h and 6 h post-exposure, the magnitude of fold-change from control was less than 4-fold for genes that met an empirical $p$-value threshold < 0.001 (Figure 4.3, dotted lines). At 3 h the Type II pyrethroids deltamethrin (DLT), cypermethrin (CYP) and cyfluthrin (CYF) had a larger number of probe sets with empirical $p < 0.001$ than at 6 h.
A majority of these probe sets were upregulated following pyrethroid exposure. The degree of change is much more pronounced for the Type II pyrethroids as compared to the Type I pyrethroids. PERM had a very small number of probe sets with an empirical $p < 0.001$ in the paired $t$-test. BIF exposure resulted in alterations in expression mainly at 6 h while TEF exposure resulted in alteration in expression mainly at 3 h. Collectively, these data demonstrate that the overall transcriptional response of the rat frontal cortex to Type II pyrethroids is qualitatively different from the response produced by the Type I pyrethroids at behaviorally equipotent doses.

LIMMA analysis of the microarray gene expression data identified a large number of probe sets with treatment related changes in expression for the Type II pyrethroids. No significant treatment related effects were observed for the Type I pyrethroids using LIMMA. A decision tree for the determination of treatment related effects on expression in the LIMMA analysis framework is given in Figure 4.4C. Unlike the paired $t$-tests shown in Figure 4.3, LIMMA analysis utilizes a false discovery rate multiple comparison correction and an adjustment factor for lowly-expressed probe sets. Figure 4.5 demonstrates three distinct patterns of significant treatment related changes in gene expression for the three type II compounds: 1) probe sets up- or down- regulated predominantly at 3 h, 2) probe sets up- or down- regulated predominantly at 6 h and 3) probe sets up or down-regulated across both time points in the study. The first pattern describes the most dramatic changes in gene expression for each of the Type II compounds. CYP exposure yielded the least number of probe sets with treatment related changes in expression ($n = 149$) followed by DLT ($n = 399$) and CYF ($n = 3742$). In the case of CYF a large number of the identified gene expression
changes (~80%) were small in magnitude with < 1.25-fold changes from control at either
time point.

Comparison of the probe sets with treatment related changes in expression for DLT, CYP and CYF, respectively, revealed a group of probe sets commonly affected by the Type II compounds (Figure 4.6A). Comparative enrichment analysis (SAFE algorithm, Barry et al. 2005) provided a quantitative measure of similarity between the gene expression profiles of the different pyrethroids in the test panel (Table 4.1). Queried lists of probe sets were based on LIMMA analysis of individual Type II compounds in the test panel and zones of similarity in the Venn diagram comparison from Figure 4.6A. Significant enrichment of the group of probe sets identified as being affected by either DLT (n = 399) or CYP (n = 149) was observed in all three Type II compounds in the study (Table 4.1). In contrast the large list of treatment affected probe sets identified for CYF (n = 3742) was not significantly enriched for the other Type II compounds. Gene lists based on the DLT-CYP, DLT-CYF and CYP-CYF unions of the Venn diagram (Figure 4.6A) were also significantly enriched for the other Type II compound not involved in forming the union.

The LIMMA analysis did not detect significant alterations in gene expression for the Type I pyrethroids. This is in contrast to the empirical \( t \)-tests illustrated in Figure 4.3 that identified a group probe sets as being altered by PERM, BIF and TEF. A heatmap visualization of probe sets commonly altered by the Type II pyrethroids demonstrates that some of the genes within this union are changed by Type I pyrethroids, albeit at lower fold-change magnitudes than observed with the Type II’s (Figure 4.6B, arrows and bracket). Results of the comparative SAFE enrichment procedures also demonstrates a similarity in the overall response of the Type I pyrethroids to the Type II pyrethroids (Table 4.1). Probe sets
with treatment related effects for DLT were enriched for PERM. Likewise, probe sets with
treatment related effects for DLT and CYP were enriched for BIF. These data demonstrate a
degree of similarity is present in the overall transcriptional response for PERM, BIF, DLT
and CYP. The large group of probe sets with treatment related effects for CYF was enriched
in the TEF treated group but not the PERM and BIF treated groups, demonstrating that the
overall transcriptional response of TEF is more similar to CYF than CYP or DLT. All of the
gene lists defined from the common overlaps of the Type II pyrethroids had significant
enrichment in all of the Type I compounds tested.

Lists of genes commonly altered by any combination of at least two Type II
pyrethroids in the microarray data sets are given in Table 4.2. The most pronounced
response to the Type II pyrethroids was the induction of a variety immediate early genes
encoding transcription factors (Junb, c-fos, Egr2, Nr4a1, Klf4 and Klf10), phosphatases
(Dusp1, Dusp5 and Dusp6) and cellular effectors (Arc). Some glucocorticoid responsive
gene transcripts were also induced (Rasd1, Gpd1 and Sgk). In addition, all three of the Type
II pyrethroids induced the expression of a small group of receptors Cxcr4, Il6r, Vipr1 and
Tnfrsf11b. Complete lists of probe sets regulated by each of the Type II pyrethroids in
available in the electronic appendices for the present study.

Six transcripts were selected for confirmation of treatment-related changes in
expression for all six pyrethroids in the test panel using qRT-PCR: Nr4a1, Arc, Gpd1, Nedd9,
Camk1g and Egr1. The first four are located in the DLT-CYP-CYF union in Figure 4.6A.
Camk1g, and Egr1 are located in the DLT-CYF, and CYP-CYF unions, respectively. A
summary of statistical analyses of qRT-PCR data for these genes is given in Appendix B,
Table 2. Expression of Nr4a1 mRNA was increased following exposure to all six
pyrethroids in the test panel. Nr4a1 induction was of much greater magnitude for the Type II pyrethroids (4 to 6-fold at 3 h) than the Type I pyrethroids (< 3-fold at 3 h). The expression pattern of Arc mRNA was similar to the expression patterns observed for Nr4a1 for all pyrethroids except lower in magnitude. Arc mRNA expression was increased with all the Type II pyrethroids on the order of 3 to 4-fold at 3 h. In contrast, Arc expression was increased only 1.5-fold at 3 h for the Type I pyrethroids. Gpd1 mRNA expression was increased for all of the Type II pyrethroids with the largest responses observed for DLT, CYF and CYP respectively. Only PERM had a significant effect on Gpd1 expression and this effect was very low in magnitude (<1.2-fold). Camk1g mRNA expression was affected most dramatically by DLT at 6 h. A smaller increase in Camk1g expression (~1.3-fold) was observed across both time points in the study for CYP and CYF (main effect of time, see Appendix B, Table 2) however no main effects of treatment were observed at p < 0.05. Nedd9 mRNA expression was increased at 3 h for DLT and CYP only, consistent with its location in the comparison in Figure 4.6A. The Type I pyrethroids had no effects on Camk1g or Nedd9 expression. Egr1 mRNA expression was increased following exposure to all three Type II pyrethroids similar to the trends in expression observed for the other immediate early genes Nr4a1 and Arc. TEF was the only Type I with significant effects on Egr1 mRNA expression. Overall, gene expression patterns measured by qRT-PCR had good concordance with the microarray data.

Two methods were implemented to identify biologically meaningful associations between the transcripts regulated by the two Type II pyrethroids: SAFE analysis (Barry et al. 2005) and Ingenuity® pathway analysis. SAFE analysis of Gene Ontology ‘molecular function’ (GOMF), ‘biological process’ (GOBP) and ‘cellular component’ (GOCC)
categories from Bioconductor v1.8 did not detect significant enrichment for any of the pyrethroids in the test panel using the Yekutieli and Benjamini (1999) false discovery rate correction at a threshold of $p < 0.1$ (data not shown). However, the overlapping GOMF categories ‘MAP kinase tyrosine-serine/threonine phosphatase activity’ (GO:0017017) and ‘MAP kinase phosphatase activity’ (GO:0044440) had an empirical $p < 0.01$ for all of the Type II pyrethroids in the panel. This is consistent with the numerous phosphatases and kinases demonstrated to be upregulated after Type II exposure (Table 4.2).

Input of genes commonly regulated by all of the Type II pyrethroids into the Ingenuity® pathway analysis software package yielded a complex interconnected network of protein interactions (Figure 4.8C). This network is comprised primarily of inducible transcription factors and protein phosphatases upregulated following Type II pyrethroid exposure and a variety of interconnected kinase complexes, growth factors and transcription factors that are constitutively expressed in neurons and not transcriptionally regulated by pyrethroids in the present study (Figure 4.8A-B). Dissection of this interconnected network into its component parts demonstrates that the phosphatases and kinases upregulated by the Type II pyrethroids primarily act upstream of the constitutively expressed interconnecting kinase complexes (Figure 4.8D). Furthermore, the constitutively expressed kinase complexes and transcription factors ($NF_{\kappa}B$, $STAT$, $CREB$) act primarily upstream of the pyrethroid inducible transcription factors (Figure 4.8E). Other pyrethroid upregulated genes ($Ier2$, $Gadd45b$, $Nedd9$, $Per1$ and $Per2$) are downstream of interconnecting transcription factors and growth factors. The peptide receptor $Vipr1$ acts upstream of $NF_{\kappa}B$ while the chemokine receptor $Cxcr4$ has a variety of upstream and downstream interactions with the constitutive kinase complexes (Figure 4.8F). Figure 4.8G-H illustrates the protein
interactions between the constitutive kinase complexes and the transcription factors or
growth factors present in the Ingenuity® network, respectively. The constitutive
transcription factors are downstream of the kinase complexes (Figure 4.8G, orange arrows)
while the kinase complexes are downstream of the growth factors (Figure 4.8H, orange
arrows).

Discussion.

The present study demonstrates that Type I and Type II pyrethroids do not produce
equivalent effects on gene transcription in the rat frontal cortex following behaviorally
equipotent doses. The expression of a large number of transcripts was affected by the Type
II pyrethroids with maximal changes in expression on the order of 4-fold from control. The
mRNA expression patterns produced by the Type II pyrethroids have features common to
DLT, CYP and CYF as well as features unique to each of the respective compounds. The
observed gene expression changes are consistent with pyrethroids producing increased
excitability of cortical circuits in vivo. Ingenuity® pathway analysis modeling yielded a
complex network of interconnecting gene expression changes consistent with increases in
neuronal excitability by the Type II pyrethroids. In addition, visual inspection of the
microarray gene expression data and comparative enrichment analysis indicated that some
components of the common Type II transcriptional response were also present in the Type I
treatment groups, albeit at lower magnitudes of change. This hypothesis was supported by
qRT-PCR analysis of a sub-set of pyrethroid regulated mRNAs.

A principal finding from this study is that Type I and Type II pyrethroids
administered at dose levels that produce equivalent effects on an apical behavior (i.e. motor
activity) cause dramatically different effects on global gene expression in the rat frontal
cortex. A large number of gene expression changes were detected with the Type II pyrethroids DLT, CYP and CYF using the LIMMA analysis framework (Figure 4.5). In contrast, LIMMA did not detect any gene expression changes with the Type I pyrethroids PERM, BIF and TEF even though empirical pair-wise contrast tests indicated that a small number of low-magnitude gene expression changes occurred following Type I pyrethroid exposure (Figure 4.3). These data are consistent with the differential effects of the two pyrethroid types on voltage-sensitive sodium channel function. Type I pyrethroids produce abnormal Na\(^+\) tail currents with time constants of decay much shorter than those produced by Type II pyrethroids (Song et al. 1996; Tabarean and Narahashi 1998; Narahashi 2000; Choi and Soderlund 2006). The longer duration Na\(^+\) tail currents produced by Type II pyrethroids maintain neurons in a more hyperexcitable state than Type I pyrethroids (Ecobichon 2001; Ray and Fry 2006). Furthermore, Type II pyrethroids produce longer trains of repetitive firing following stimulus than the Type I pyrethroids (Vijverberg and Van den Bercken 1990). The present data suggest that Type II pyrethroids produce a greater degree of neuronal excitation in the cortex than the Type I pyrethroids at the administered doses examined which would in turn produce more dramatic changes in the expression of genes regulated by neuronal activity.

The differences in gene expression profiles between the two pyrethroids types may also reflect differential activation of secondary molecular targets sites that control gene transcription. For example, Ca\(^{2+}\) is an intracellular second messenger capable of triggering \textit{de novo} gene transcription (Schulman and Roberts 2003). Symington and Clark (2007) have demonstrated an increase in Ca\(^{2+}\) influx into rat brain synaptosomes following both deltamethrin (Type I) and cismethrin (Type II). Cismethrin-stimulated Ca\(^{2+}\) influx was
dependent upon Na\textsuperscript{+} channel mediated depolarization while deltamethrin-stimulated Ca\textsuperscript{+2} influx was Na\textsuperscript{+} channel independent. In addition, the concentration-response curve for deltamethrin-stimulated Ca\textsuperscript{+2} influx is to the left of that for cismethrin, indicating a smaller concentration of the Type II pyrethroid is required to produce the response. It is possible that at the dose levels used in the present study, the Type II pyrethroids are more potent activators of a secondary molecular target(s) that mediate gene transcription than the Type I pyrethroids. These actions could be independent of both pyrethroid effects at VSSCs and the behavioral response (i.e. decreased motor activity), which may explain why the doses used here produce the same magnitude of behavioral change for the two pyrethroid types but do not produce the same magnitude of gene expression changes.

An additional alternative is that pharmacokinetic differences in the distribution or metabolism of Type I and Type II pyrethroids may be contributing to the divergent effects on global gene expression observed here. Comparison of pharmacokinetic (PK) data available in the literature for DLT and PERM do not support this conclusion (PK models for CYP, CYF, BIF and TEF in the rat are not presently available). Both DLT and PERM accumulate in the frontal cortex following an acute oral dose (Anadon 1991; Anadon 1996). Furthermore, brain concentrations of both Type I and Type II pyrethroids correlate with changes in behavior at high doses (Gray et al. 1980; Rickard and Brodie 1985) and doses compatible with those used in this study (Scolon et al. in preparation). Lastly, pyrethroids all have different efficacies for interacting with different VSSC isoforms (Soderlund et al. 2000; Choi and Soderlund 2006). In \textit{in vitro} studies of neuronal function, different pyrethroid types produce disparate effects on the excitable properties of the same neuronal cell populations and different neuronal subtypes respond differently to the same pyrethroid
(Tatebayashi and Narahashi 1994; Tabarean and Narahashi 1998; Grosse et al. 2002; Meyer and Shafer 2006; Meyer et al. 2007). Therefore, since different VSSC isoforms and neuronal subtypes are heterogeneously expressed throughout central nervous system tissues, the unique effects of the individual pyrethroid types on channel function may be the critical factor contributing to the differential gene expression patterns.

The transcriptional response of the frontal cortex to the Type II pyrethroids DLT, CYP and CYF contains some genes that are commonly regulated by multiple Type II pyrethroids in the test panel and some genes that are uniquely regulated by the individual Type II pyrethroids (Figure 4.6A). These data support that all three of the Type II pyrethroids in the test panel elicit similar changes in the expression of a sub-set of genes while also producing changes in expression unique to the different Type II compounds. The comparative SAFE enrichment analysis also supported this conclusion and demonstrates that the transcription response of DLT and CYP is the most similar. Differences in chemical structure between the three Type II pyrethroids in the panel may be responsible for mediating the differences in the global transcriptional response of DLT, CYP and CYF. Structurally, DLT and CYP differ only in the identity of halogen groups on the cyclopropane carboxylic acid moiety of the compounds: bromines for DLT and chlorines for CYP (Figure 4.1). CYF differs from CYP only by the addition of a fluorine group to the aromatic alcohol moiety (Figure 4.1). These small differences in chemical structure can have impacts on the duration of VSSC modification and adverse outcomes on the behavioral level (Verschoyle and Aldridge 1980; Choi and Soderlund 2006). Differences in stereoisomer ratios between the Type II pyrethroids may also explain the divergences observed in global gene expression profiles. The preparation of DLT used is composed exclusively of the ‘more toxic’ cis-(αR)
stereoisomer whereas CYP and CYF are heterogenous mixtures of cis- and trans- α-S and α-R stereoisomers (Soderlund 1985; Ray 2001; Soderlund et al. 2002). Definitive conclusions regarding the impact of chemical modifications or stereochemistry on pyrethroid induced gene expression can not be made without systematic comparison of isomerically pure preparations of the test compounds. However, the present data clearly demonstrate that there is a specific suite of genes commonly regulated by Type II pyrethroid compounds regardless of isomeric composition.

The mechanistic relationship(s) between acute pyrethroid action at ion channels and the genes altered by Type II pyrethroids is currently unclear. However, the present data are consistent with the previous findings (Harrill et al. submitted). Both studies failed to detect any changes in gene expression corresponding to the primary molecular targets of pyrethroids (i.e. VSSCs, Ca$^{2+}$ channels, Cl$^{-}$ channels). On the other hand, some of the transcripts within the suite of genes commonly regulated by the Type II pyrethroids are known to be upregulated in response to excitatory stimulation. This supports the hypothesis that the Type II pyrethroids DLT, CYP and CYF produce neuronal excitation within the frontal cortex in the present study. The expression of the immediate early (IEG) transcription factors c-fos, JunB, Egr1 and Nr4a1 have been shown to be upregulated by a variety of excitatory stimuli (Hazel et al. 1991; Morgan and Curran 1991; Fields et al. 1997; Hughes et al. 1999; Patra et al. 2004) and are also upregulated by Type II pyrethroids in the present study. In addition, the expression of the phosphatases Dusp1, Dusp5 and Dusp6 and the cytoskeletal protein Arc that are upregulated by DLT, CYP and CYF in the present study are also induced following neuronal excitation (Gass et al. 1996; Muda et al. 1996; Hevroni et al. 1998; Kodama et al. 2005; Guzowski et al. 2006; Machado et al. 2008). Another component
of the common Type II response are the genes *Rasd1* and *Gpd1* which are both regulated by activation of intracellular glucocorticoid receptors by the hypothalamic-pituitary-adrenal axis (Baughman et al. 1997; de Kloet et al. 1998; Brogan et al. 2001). The upregulation of *Gpd1* indicates that transcriptional responses of glial cells may be included in the overall global expression profiles for pyrethroids in the frontal cortex, given that this protein is expressed exclusively in oligodendrocytes (Leveille et al. 1980). In addition, *Sgk* has been shown to be upregulated following both glucocorticoid receptor activation and activation of post-synaptic excitatory glutamate receptors (Lee et al. 2003; Webster et al. 2003) which supports a role for both neuronal excitation and glucocorticoid stimulation in the transcriptional response of the frontal cortex to pyrethroids. These data are consistent with previous studies that demonstrate induction of activity-regulated genes and increases in circulating corticosterone following acute pyrethroid exposure (Hassouna et al. 1996; de Boer et al. 1998; Wu and Liu 2003). Even though the mechanistic linked between pyrethroid action at the ion channel and the present gene expression is unknown, these data do provide insight into some of the intracellular signaling pathways that may be stimulated by acute pyrethroid action in vivo.

Ingenuity® pathway analysis of genes commonly affected by the Type II pyrethroids yielded an interconnected network of protein interactions that is also consistent with the induction of hyperexcitability by pyrethroids in the frontal cortex. This network is comprised of the IEG transcription factors, phosphatases and effector proteins commonly upregulated by the Type II pyrethroids as well as constitutively expressed kinase and transcription factor complexes (Figure 4.8C). Systematic deconstruction of this interactive network supports a model of acute pyrethroid action that includes stimulation of constitutive kinase signaling cascades and subsequent activation of constitutive transcription factors followed by de novo
expression of IEG phosphatases and kinases that deactivate the excitatory signaling pathways, effector proteins that may impact cellular function and IEG transcription factors that can regulate subsequent changes in the expression of additional effector proteins (Figure 4.9). Increased neuronal excitation results in the activation of Akt, Jnk, MAPK, p38 MAP, MEK and Pkc (Rosen et al. 1994; Vanhoutte et al. 1999; Perkinton et al. 2002; Brager et al. 2003; Chen et al. 2003; Vaillant et al. 2003; Corvol et al. 2005; Pezet et al. 2005) which are the connecting nodes of the Ingenuity® network (Figure 4.8C). Dusp1, Dusp5, Dusp6 and Ptpn1 may then act to dephosphorylate and deactivate these kinases, and to turn off the excitatory stimulus in a transcription dependent feedback loop. The activated kinase complexes are also upstream of de novo expression of the IEG transcription factors induced by the Type II pyrethroids. The model supports a role for the constitutive transcription factors STAT, CREB and NFκB in connecting the excitatory responses of the kinase networks to the pyrethroid induced de novo gene expression changes. These three proteins are downstream of the putatively activated kinase complexes and upstream of the transcriptional responses elicited by the Type II pyrethroids (Figure 4.8D and 4.8E, Wooten 1999; Davis et al. 2000; Kaltschmidt et al. 2002). Ingenuity® also incorporated the growth factors Fgf, Pdgf and Tgfb into the network based on upstream effects on the inducible components of the Type II pyrethroid response. However, there are no data available that support a role for these genes in the biological activity of pyrethroids in vivo. At this time, the effect of de novo gene expression induced by pyrethroids on downstream cellular functions is unclear. Additional studies are needed to investigate the activation of the signaling pathways putatively identified in the present study in response to pyrethroids.
Several lines of evidence within that current study suggest that components of the transcriptional response commonly elicited by the Type II pyrethroids (Table 4.2, far right column) was also be present in the Type I treatment groups, even though LIMMA analysis did not detect them. First, pair-wise comparisons of PERM, BIF and TEF treated groups to vehicle controls indicated that the expression of a small group of probe sets was in fact changed (Figure 4.3). Second, visual inspection of the expression profiles for genes commonly affected by the Type II pyrethroids demonstrated that a small sub-set of genes had qualitatively similar changes in expression between pyrethroid types, although the response produced by the Type I’s was lower in magnitude (Figure 4.6B, brackets). Lastly, comparative SAFE enrichment analysis (Table 4.1) demonstrated that gene lists derived from the overlapping portions of the Type II transcriptional responses were significantly enriched in the PERM, BIF and TEF treatment groups. Similarities in the transcriptional response to Type I and II pyrethroids has been previously reported (Harrill et al. submitted). These observations support that the multiple-test correction employed by LIMMA may have been too conservative for the purpose of detecting the small number of low-magnitude gene expression changes observed in the Type I treatment groups. Alternatively, the use of motor function data (Wolansky et al. 2006) to define equipotent dose levels may have overestimated the potency of Type I pyrethroids at the neuronal membrane. This latter point is consistent with the smaller effects observed with the Type I pyrethroids in this study. Dose-response data on gene expression for Type I pyrethroids using higher administered doses would test this hypothesis.

Quantitative RT-PCR analysis performed on a group of transcripts commonly regulated by multiple Type II pyrethroids in the microarray experiment are consistent with
the expression patterns observed in the microarray study and also support that a small number activity-regulated genes are regulated in response to the Type I pyrethroids. *Arc* and *Nr4a1* IEG expression was significantly changed by all six pyrethroids in the test panel (Figure 4.7). *Egr1* and *Gpd1* mRNA was upregulated by all the Type II pyrethroids and by PERM and TEF respectively. *Nedd9* expression was upregulated by DLT and CYP while *Camk1g* was significantly increased only by DLT. The changes in *Arc* and *Nr4a1* mRNA expression for the Type I pyrethroids were of smaller magnitude than those observed with the Type II pyrethroids and is consistent with a lesser degree of neuronal activation in the cortex for the doses PERM, BIF and TEF used here as compared to DLT, CYP and CYF. In a previous study (Harrill et al. *submitted*) a dose of 100 mg/kg PERM produced increases in *Egr1*, *Gpd1* and *Camk1g* comparable to the 3 mg/kg dose of DLT used in the present study. The 100 mg/kg PERM dose examined was slightly more efficacious for decreasing ambulatory motor activity than the 3 mg/kg DLT dose (ED$_{50}$ vs. ED$_{30}$, Wolansky et al. 2006). This is consistent with the hypothesis stated above that suggests higher doses of Type I pyrethroids will produce similar effects to the Type II pyrethroids observed in the present study. These data do not support that the frontal cortex is insensitive to the effects of Type I pyrethroids in terms of *de novo* gene transcription.

The lack of effect of Type I pyrethroids on *Camk1g* mRNA is inconsistent with previous studies (Harrill et al., *submitted*, Harrill and Crofton, *in preparation*). In those studies an ED$_{30}$ dose of DLT and either an ED$_{30}$ or ED$_{50}$ doses of PERM (based on Wolansky et al. (2006) motor activity data) were sufficient to produce an upregulation of *Camk1g* mRNA. In addition, in these previous studies, the *Camk1g* mRNA response to PERM was less robust that that observed with DLT. In the present study, an ED$_{30}$ dose of
DLT again resulted in an increase in *Camk1g* mRNA expression, whereas an ED$_{30}$ dose of PERM caused no effect. The reason for this disparity is unclear. The PERM used in the present study was taken from the same lot of technical grade product as used in the two previous studies. Therefore, differences in isomer composition and purity cannot account for the disparate results. Alternatively, differences in the metabolic rates for PERM between the cohorts of animals used across studies may account for the difference. The rats used in the present study may have had a greater capacity for metabolizing PERM than the rats used in the previous studies, therefore resulting in a lower target tissue concentration at a comparable administered dose level and no appreciable increase in *Camk1g* mRNA.

Collectively, across studies the data indicate that *Camk1g* mRNA expression is affected by pyrethroids with DLT being the most potent for producing this effect. *Camk1g* mRNA expression in cortical neurons is responsive to changes in neuronal firing patterns and internal Ca$^{+2}$ trafficking (Xiang et al. 2007). The greater relative potency of DLT for increasing in *Camk1g* mRNA expression may be due to the longer duration Na$^+$ tail currents, and consequently greater increase in neuronal excitability, produced by DLT as compared to other pyrethroids (Vijverberg and Van den Bercken 1990; Choi and Soderlund 2006). Alternatively, DLT may activate a secondary molecular target that controls *Camk1g* expression, such as a voltage-sensitive Ca$^{+2}$ channel, at lower concentrations than other pyrethroids (Symington and Clark 2007). Further work is needed to substantiate these hypothesis as a formal link between pyrethroid action at the neuronal membrane, increased internal Ca$^{+2}$ concentrations and increased *Camk1g* mRNA expression has not been established.
In conclusion, the present data demonstrate that the Type II pyrethroids produce more pronounced changes in activity-regulated gene expression than Type I pyrethroids in the frontal cortex of rats orally exposed to behaviorally equipotent doses. The data also provide predictions for some intracellular signaling pathways that may be affected directly downstream of pyrethroids actions at the neuronal membrane.

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**Acknowledgements.**

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Table 4.1. *Enrichment scores for gene-of-interest lists derived from Venn Diagram comparison.*

<table>
<thead>
<tr>
<th>Type</th>
<th>n</th>
<th>DLT</th>
<th>CYP</th>
<th>CYF</th>
<th>PERM</th>
<th>BIF</th>
<th>TEF</th>
</tr>
</thead>
<tbody>
<tr>
<td>DLT-Limma</td>
<td>399</td>
<td>n/a</td>
<td>0.0001</td>
<td>0.0009</td>
<td>0.0345</td>
<td>0.0314</td>
<td>0.1433</td>
</tr>
<tr>
<td>CYP-Limma</td>
<td>149</td>
<td>0.0001</td>
<td>n/a</td>
<td>0.0022</td>
<td>0.0689</td>
<td>0.0030</td>
<td>0.0854</td>
</tr>
<tr>
<td>CYF-Limma</td>
<td>3742</td>
<td>0.1246</td>
<td>0.3499</td>
<td>n/a</td>
<td>0.0874</td>
<td>0.1632</td>
<td>0.0046</td>
</tr>
<tr>
<td>DLT-CYP Union</td>
<td>91</td>
<td>n/a</td>
<td>n/a</td>
<td>0.0003</td>
<td>0.0345</td>
<td>0.0060</td>
<td>0.0258</td>
</tr>
<tr>
<td>DLT-CYF Union</td>
<td>216</td>
<td>n/a</td>
<td>0.0001</td>
<td>n/a</td>
<td>0.0345</td>
<td>0.0311</td>
<td>0.0258</td>
</tr>
<tr>
<td>CYP-CYF Union</td>
<td>85</td>
<td>0.0001</td>
<td>n/a</td>
<td>n/a</td>
<td>0.0348</td>
<td>0.0031</td>
<td>0.0258</td>
</tr>
<tr>
<td>DLT-CYP-CYF Union</td>
<td>71</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>0.0345</td>
<td>0.0060</td>
<td>0.0107</td>
</tr>
</tbody>
</table>

Gene lists derived from the LIMMA analysis Venn Diagram (Figure 4.6a) were analyzed for enrichment by SAFE analysis across all six compounds in the study. Prior to analysis microarray expression values were re-centered to the combined mean of 3 h and 6 h controls to eliminate main effects of time (Appendix B, Figure 1). The local statistic for the SAFE analysis is based on a multi-factor ANOVA of mean centered expression values. Global statistics are based on a Wilcoxon rank sum test. The threshold for significant enrichment was set at an FDR < 0.05 (highlighted in bold). n/a = not analyzed; compounds upon which a particular gene-of-interest list is based were not tested for enrichment for that list.
Table 4.2. *Genes changed by Type II pyrethroid exposure.*

<table>
<thead>
<tr>
<th>Genes Changed</th>
<th>DLT-CYP Union</th>
<th>DLT-CYF Union</th>
<th>CYP-CYF Union</th>
<th>DLT-CYP-CYF Union</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Transcription / Nuclear Factors</strong></td>
<td>Vgll4</td>
<td>Cebpb, Tsc22d3, Ets2, Mybbp1a, Madh3, Mcyn</td>
<td>Egr1, Tcfcp212</td>
<td>Klf10, Junb, Nab2, c-fos, Nr4a1, Id1, Hes1, Klf4, Egr2</td>
</tr>
<tr>
<td><strong>Phosphatases</strong></td>
<td>Acpl2</td>
<td>Ptpru</td>
<td>Dusp1, Dusp5, Dusp6, Ptpn1</td>
<td></td>
</tr>
<tr>
<td><strong>Kinases</strong></td>
<td>Map2k3</td>
<td>Camk1g, Mertk, Pdk4, Pim3, Uae1</td>
<td>Sgk, Snf1lk, Sbk, Tribl</td>
<td></td>
</tr>
<tr>
<td><strong>Cytoskeletal/Structural Proteins</strong></td>
<td>Cryab, Pxn, Pkp2</td>
<td></td>
<td>Arc, Nedd9, Lims2</td>
<td></td>
</tr>
<tr>
<td><strong>Metabolic Proteins</strong></td>
<td>Fmo2, Fmo3, Lfng, Xdh, Sult1a1, Hyal2</td>
<td></td>
<td>Gpd1, Gfpt2, Hs3st1</td>
<td></td>
</tr>
<tr>
<td><strong>Ligands / Receptors</strong></td>
<td>Nppc</td>
<td>Sstr2, Chrhm4, Adipor2</td>
<td></td>
<td>Cxcr4, Il6r, Vipr1, Tnfrsf11b</td>
</tr>
<tr>
<td><strong>Extracellular Matrix Proteins</strong></td>
<td>Vwa1, Prss11, Lcn7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Ion Channels / Ion Transport</strong></td>
<td>Gjb6, Cacng2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Transporters</strong></td>
<td>Slc21a14, Slc2a1, Slc25a25, Slc39a1</td>
<td></td>
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</table>
## Table 4.2. continued

<table>
<thead>
<tr>
<th>Cellular Stress Response</th>
<th>Hspb1</th>
<th>Bcl2l1, Ndg1, Rbm3</th>
</tr>
</thead>
</table>

### Adaptor Proteins<sup>a</sup>

<table>
<thead>
<tr>
<th></th>
<th>Homo1</th>
<th>Ralgds, Mgl1, Errfi1, Lrg1, Rin3, Fkbp51, Sle9a3r2, Cdkn1a, Cnksr3, Nfkbia, Bag3, Arhgap7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Other / Unknown Functions</td>
<td>Ctrl, Smpdl3b, Arrdc2, Dega, Clec14a, Prr, Srxn1, Nid67, LOC684871, LOC690516, LOC683687, RGD1311086, RGD1308276, RGD1359349, RGD1311086, RGD1304790</td>
<td></td>
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</table>

### Predicted Protein Sequences

<table>
<thead>
<tr>
<th></th>
<th>Ctnbp2nl, Rasgef1c</th>
<th>Rasgrp3, Pnpla2, Sesn1, Cbr3, Mk1, Asah3l, Spsb1, B3galt5, Cables1, Map3k6, Bcl6, Cd163, Asph, Rundc1, Pla2g3, Usp43, Cdc42ep4, Pwwp2, Mrc1, RGD1310714, RGD1561512, RGD1559797, RGD1306323</th>
</tr>
</thead>
</table>

### Number of ESTs

<table>
<thead>
<tr>
<th></th>
<th>8</th>
<th>39</th>
<th>9</th>
<th>12</th>
</tr>
</thead>
</table>

<sup>a</sup>Columns were defined from different zones of similarity shown in Figure 4.6A.  
<sup>b</sup>Proteins that directly associate with and affect the function of another protein without phosphorylation or dephosphorylation.
Figure 4.1. Chemical structures of pyrethroids.
Figure 4.2. Pyrethroid effects on motor activity. Data replotted from Wolansky et al. (2006). Ambulatory motor activity expressed as percent change from vehicle treated control ± standard error (y-axis) versus administered dose of pyrethroids (x-axis). Type II pyrethroids plotted in gray, Type I pyrethroids plotted in black. The calculated ED$_{30}$ for decreased motor activity for each compound is given in the inset. These are the administered doses used in the present study.
**Figure 4.3.** Volcano plots of pair-wise comparisons of microarray data. Each panel plots the empirical $p$-value from a pair-wise $t$-test of treated groups to their corresponding time-matched vehicle controls groups (y-axes) versus fold change from control (x-axis). Type II pyrethroids are in the upper panels while Type I pyrethroids are in the lower panels. 3 h treatment groups are in rows 1 and 3. 6 h treatment groups are in rows 2 and 4. Dashed lines represent an empirical $p$-value threshold of $p < 0.001$. All probe sets present on the Affymetrix Rat 230_2 array are shown in each panel.
Figure 4.4. Linear Models for Microarray Data (LIMMA) analysis scheme. (A) Condition matrix for each of the compounds examined in the study. Two treatments (control and pyrethroid) and two times (3 h and 6 h) comprise the 2-by-2 matrix. (B) Contrasts made in the LIMMA analysis for each probe set. Treatment groups involved in each contrast are listed in brackets. The structure is identical to a two-way ANOVA model. (C) Flowchart for the determination of treatment related changes in expression by LIMMA analysis. Decisions for each contrast are made based on a significance threshold of $p < 0.1$ for Yekutieli-Benjamini (1999) adjusted $p$-values. Gene expression values for each pyrethroid were examined individually within this framework.
Figure 4.5. Results of LIMMA analysis for Type II pyrethroids. Each panel shows gene expression changes as a function of fold-change from control (y-axes) across time (x-axes) for probe sets with treatment related changes in expression for DLT (blue tabbed panels), CYP (red tabbed panels) and CYF (orange tabbed panels). Branches within the diagram correspond to the decisions regarding each contrast outline in the flowchart in Figure 4.4C. Tabs with black numbers in the top two or last row of the diagram represent the number of probe sets that met the particular condition. Probe sets with > 1.25-fold changes in expression for either the 3 h or 6 h condition are shown in blue and correspond to the blue numbers in each panel. Probe sets with no fold changes in expression >1.25 for either the 3 h or 6 h condition are shown in gray and correspond to the gray numbers in each panel.
Figure 4.6. Comparison of gene expression changes across compounds. (A) Venn diagram comparing treatment related changes in gene expression for the Type II pyrethroids deltamethrin, cypermethrin and cyfluthrin. (B) Heatmap displaying patterns of gene expression changes for the 71 probe sets commonly regulated by the Type II pyrethroids. Columns are individual probe sets. Rows are treatment groups. Areas of apparent similarity between the Type II and Type I compounds are marked with arrows and brackets. Tile coloring is based on the mean fold change from control for each of the treatment conditions.
**Figure 4.7. qRT-PCR results.** Each panel shows changes in gene expression measured with TaqMan® qRT-PCR for Nr4a1, Arc, Gpd1, Camk1g, Nedd9 and Egr1. Each gene of interest was examined for each pyrethroid in the test panel. Data are expressed as $2^{-\Delta\Delta CT}$ values ± standard error (y-axes) across time (x-axes) as described in the Methods section. Colored boxes beside each gene name correspond to the area of the Venn Diagram in Figure 4.6A in which each transcript is found. Compound abbreviations listed under each gene symbol denote that a significant effect of treatment was observed for that individual pyrethroid ($p < 0.05$). Statistical analyses of these data are described in the Methods section and results are given in Appendix B, Tables 2.
Figure 4.8. Ingenuity® pathway analysis of genes commonly affected by Type II pyrethroids. The 71 probe sets commonly affected by all the Type II pyrethroids in the microarray data was input into the Ingenuity® pathway analysis software package and yielded the interconnected network shown in panel C. (A) List of the pyrethroid-sensitive genes present in the network. Coloring of these elements is based on the expression pattern observed with deltamethrin: red = upregulated, blue = downregulated. (B) List of connecting nodes present in the network. (D-F) Systematic deconstruction of the interaction network in Figure C. Associations between the connecting nodes of the full network removed for clarity in panels D-F. The network is separated into an inducible phosphatase/kinase component (D), an inducible transcription factor component (E) and other regulated proteins component (F). Note in panel D that inducible phosphatases act upstream of the constitutive kinases (arrows pointed to the right) and in panel E constitutive kinases and transcription factors act upstream of inducible transcription factors (arrows radiating inward). (G-H) The interconnections of the constitutive kinase complexes are given in the absence of pyrethroid-responsive genes for clarity. Interconnections between kinase complexes and transcription factors (orange arrows pointing out) and kinase complexes and growth factors (orange arrows pointing in) are given in panels G and H.
Figure 4.9. Schematic of putative pyrethroid gene regulatory network. Putative intracellular signaling cascades impacted by pyrethroids as indicted by Ingenuity® analysis. A = activation, TR = transcription, TL = translation, D = deactivation, M = translocation / movement.
Works Cited


Discussion and Future Directions

Chapter 5

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The present research project was undertaken to address a fundamental data gap in the toxicological profile of pyrethroid insecticides: i.e. changes in gene expression that occur downstream of the pharmacological interaction of these agents with voltage-sensitive ion channels. The acute neurotoxic effects of pyrethroids manifest at the whole organism level as a result of pharmacological disruption of neuronal firing (Narahashi 2001; Soderlund et al. 2002; Ray et al. 2005). A vast literature in the fields of molecular biology and neuroscience demonstrate that increased neuronal excitability produce changes in gene expression. These changes in gene expression are thought to mediate adaptive responses of neurons to excitatory stimuli in both adult and developing animals (Finkbeiner and Greenberg 1998; Zhang and Poo 2001; Wong and Ghosh 2002; Zito and Svoboda 2002; Miller and Kaplan 2003; Uesaka et al. 2006; Uesaka et al. 2005; Kullman and Lamsa 2007). Yet, the effects of pyrethroids on neuronal gene expression downstream of actions at the neuronal membrane had not been characterized.

The overall goal of the present research was to test the hypothesis that pyrethroids cause changes in the expression of activity-regulated gene transcripts in the central nervous system in dose ranges surrounding the threshold for acute neurotoxic effects. The present studies: 1) identified activity-regulated gene expression changes following acute pyrethroid exposure, 2) identified some intracellular signaling pathways that may be activated by pyrethroids, and 3) demonstrated that neuronal branching morphogenesis is affected by pyrethroid exposure in vitro. The latter finding may represent a novel neurotoxic effect of pyrethroids.

The gene expression studies conducted in Chapters 2 and 4 of this work identified a suite of activity-regulated genes as being sensitive to pyrethroids including \(c-fos,\) \(Arc,\) \(Nr4a1,\)
Egr1, Dusp1, Dusp5, Dusp6 and Camk1g. The majority of these findings are novel to the field of pyrethroid research and consistent with the neuronal hyperexcitability produced by pyrethroids. These changes in gene expression occur at or below doses that produce minimal effects on neurobehavior in the whole animal (Bloom et al 1983; Crofton and Reiter 1984; Peele and Crofton 1987; McDaniel and Moser 1996; Wolansky et al. 2006). The activity-regulated transcripts identified as being responsive to pyrethroids are a ‘molecular tool’ useful in addressing a critical gap in the field of pyrethroid research; do different pyrethroids activate the same neuronal circuits at doses that produce minimal effects on neurobehavior?

The neurobehavioral endpoints affected by pyrethroids are apical measures of nervous system function and can be affected by a wide variety of chemicals with diverse toxicological mechanisms (Davis 1986; Moser and MacPhail 1990; Crofton et al. 1991; Crofton and MacPhail 1996). It is possible that at moderately effective dose levels, Type I and Type II pyrethroids activate different neuronal circuits or different neuronal sub-types to elicit the same overall effect on apical neurobehavioral endpoints. In support of this, in vitro studies have demonstrated that different pyrethroid types produce disparate effects on the same neuronal cell populations and that neurons with different cellular phenotypes respond differently to the same pyrethroid (Tatebayashi and Narahashi 1994; Tabarean and Narahashi 1998; Stucky and Lewin 1999; Grosse et al. 2002; Wu and Pan 2004; Meyer and Shafer 2006; Meyer et al. 2008). In addition, the present data from Chapter 4 demonstrate that activity-regulated gene expression in the rat cortex is less intense following Type I pyrethroid exposure as compared to Type II pyrethroids at low doses that produce the same quantitative effect on motor function. The pyrethroid-regulated gene signatures defined in the present study may be used as cellular ‘markers-of-activation’ to compare the excitatory effects of
pyrethroids, both across brain regions and across different compounds. Data on activation of specific neuronal circuits in the lower end of the pyrethroid-dose effect range would aid in the mechanistic grouping of different pyrethroids in the context of a cumulative risk assessment framework (EPA 2002).

The mapping of activated neuronal circuits with immediate early genes has been used to study both behavior in untreated animals and neurotoxicity produced by a variety of compounds (Vendrell et al. 1991; Vendrell et al. 1992; Andre et al. 1998; Guzowski et al. 1999; Storey et al. 2002; Caravajel et al. 2005). Guzowski et al. (2005) demonstrated the utility of fluorescent in situ hybridization (FISH) in identifying neurons activated by an excitatory stimuli using an immediate early mRNA as the marker of effect. The immediate early genes identified in the present study could be used as markers of effect with the FISH method to examine the neuronal circuitry activated by pyrethroids in an acute dosing model. Future experiments could include a multi-factorial design that examines pyrethroid-induced neuronal activation at several doses surrounding the threshold for acute behavioral effects, several sampling times that match the onset and peak of neurobehavioral effects and several different pyrethroids for comparison of effects across the chemical class. Serial sectioning of whole brains would be used to provide a comprehensive map of neuronal cell bodies activated by pyrethroids throughout the different brain regions at different times and doses. The commercial availability of fluorophores that excite at different wavelengths also allows for multicolor FISH. Different colored fluorophores could be used to label the immediate early gene marker and other mRNA species specific to neurons with different molecular phenotypes (such as those coding for specific neurotransmitter receptors or ion channels). This would allow determinations of whether different neuronal populations within the brain
are activated by different pyrethroids or at different pyrethroid doses. The type of approach described here would address potential mechanistic differences in the Type I and Type II action at the cellular level.

From a molecular biology standpoint, the data on pyrethroid-regulated gene expression in the present study support a model for the intracellular effects of these compounds at the protein level. This putative signaling network includes the activation of constitutive kinase pathways (PI3K/Akt, Jnk, p38 MAPK, ERK/MEK, Pkc), subsequent activation of CREB, NFκB and STAT transcription factors and finally induction of immediate early gene phosphatases, transcription factors and effectors. These pyrethroid induced genes terminate stimulation of the kinase pathways, mediate subsequent gene transcription events and may augment downstream neuronal functions, respectively (see Chapter 4, Figure 4.9). The pyrethroid effects on immediate early gene expression are empirically measured in the present studies, however the activation of the constitutive kinase complexes and transcription factors in this network are speculative at this point. Future experiments examining the phosphorylation states, kinase activities and transcriptional activities of these proteins in response to pyrethroids are required to validate the proposed intracellular signaling network.

Predictions regarding the role of immediate early gene expression and the putative intracellular signaling networks as it relates to pyrethroid neurotoxicity are difficult. Some of genes upregulated by pyrethroids in the present studies (Arc, Egr1), as well as some components of the predicted pyrethroid signaling network (MAPK, p38 MAPK, Jnk, CREB, NFκB) are essential to the process of long-term potentiation and memory consolidation in the brain (Bozon et al. 2003; Li et al. 2005; Miyamoto 2006; O’Mahoney et al. 2006; Plath et al. 2006; Liu et al. 2007; Toyoda et al. 2007). Subsequently, abnormal activation of these
pathways by pyrethroids may interfere with memory formation and cognitive recall. In support of this, a limited number of \textit{in vivo} studies report effects of acute pyrethroid exposure on learned behavior in rats using operant response tasks (Bloom et al. 1983; Peele and Crofton 1987; Stein et al. 1987). A consistent decrease in operant response rates was observed for numerous pyrethroids and indicates that these compounds either interfere with cognitive recall of a learned response or, alternatively, decrease motivation for completing a reward-driven task. If protein phosphorylation and activation studies confirm the putative findings from the gene array pathway analysis, then investigation of low dose pyrethroid-induced effects on working memory (learning) and reference memory (retention) with classical behavioral test paradigms (Eckerman and Bushnell 1992) and correlation of those effects with changes in biochemical endpoints would be a sensible follow-up study.

Of all the findings in the present study, the observation that pyrethroids can affect neuronal branching morphogenesis \textit{in vitro} has the highest potential impact on human health and the field of pyrethroid toxicology. The basis for testing whether or not pyrethroids affected this developmental process came from functional category level analysis of the microarray data presented in Chapter 2 of this work. The \textit{in vitro} model used herein revealed that pyrethroids increased neurite branching but not length. This finding is novel and in contrast to the few available reports of pyrethroid effects on neuronal morphology (Nandi et al. 2006; Tran et al. 2006; Weeks and Perez 2006). The present data predict that pyrethroids may affect neuronal branching \textit{in vivo} in both the adult and developing nervous system. Repeated abuse of the stimulant drugs cocaine and amphetamine have been shown to produce increased dendritic branching in the adult cortex, which has been putatively linked to impairment of cognitive function and development of addiction (Robinson and Kolb 1999;
Robinson et al. 2001). Repeated exposures to pyrethroids may cause the same types of effects. In addition, neurons undergo extensive dendritic branching prior to the formation of synaptic contacts in the developing nervous system (Wong and Ghosh 2002; Jan and Jan 2003; Libersat and Duch 2004; McAllister et al. 2004). Pyrethroids also have the potential to affect this developmental process, based on the present in vitro findings.

The potential for pyrethroids to act as developmental neurotoxicants has been recently reviewed (Shafer et al. 2005). To date, the database on pyrethroid developmental neurotoxicology includes investigations of acetylcholine receptor density, neurotransmitter levels and motor behavior in adult rats and mice exposed during gestational or early post-natal life (Eriksson and Nordberg 1990; Eriksson and Fredriksson 1991; Ahlbom et al. 1994; Talts et al. 1998a; Lazarini et al. 2001). The present data argue that changes in neuronal microstructure and network connectivity should also be examined in laboratory animal models exposed to pyrethroids during the critical period of neuronal growth and branching.

Gene expression data provides a number of attractive candidate molecules that may mediate the pyrethroid effects on branching morphogenesis. These include Camk1g, Cxcl12 and its cognate receptor Cxcr4, Notch and β-catenin all of which control some aspects of neuronal branching (Redmond et al. 2000; Yu and Malenka 2003; Pujol et al. 2005; Wayman et al. 2006; Takemoto-Kimura et al. 2007). An immediate future direction would be to confirm the effects on branching morphology observed in vitro in an in vivo model. A morphological examination of neuronal dendritic fields following pyrethroid exposure in both early life and in adulthood would serve to confirm or refute the present observations. If pyrethroids are shown to impact neuronal branching in vivo, then the gene candidates
identified in the present studies could be used as starting point to examining the molecular mechanisms that control this response.

In summary, the present research provides important findings regarding the effects of pyrethroids on gene expression in the brain, an area of pyrethroid research that had not been extensively studied previously. The results of these studies supported the hypothetical model of pyrethroid effects on gene expression outlined prior to experimentation. In addition, these data demonstrated that different pyrethroids induce changes in a similar sub-set of activity regulated genes and facilitated predictions regarding the activation of intracellular signaling cascades and functional effects in neurons downstream of the pharmacological actions of pyrethroids at the neuronal membrane. Finally, one of the functional effects predicted by the gene expression studies was confirmed in an *in vitro* model of neuronal morphogenesis.


Appendix A. Chapter 2 Supplementary Material
### Appendix A, Table 1. Comparison of mean coefficients of variation (CV) between GCOSv1.2 and RMA microarray expression summaries.

For each expression summary calculation method, all 31,042 probe sets present on the Affymetrix Rat 230 2.0 GeneChip® array were sorted based on the mean expression summary within the control group and divided into equally sized percentile ranges in ascending order. CV’s were calculated for each individual probe set within each dose group. The mean CV for each percentile range was then calculated across probe sets for each dose group. Expression summaries calculated using RMA consistently reduces the variability of the expression summaries across the entire data set when compared to GCOSv1.2. A dramatic decrease in variability is observed in the lower 50% of the data set.

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*taken from Applied Biosystems web-site.

Appendix A. Figure 1. Identification of dose-responsive probe sets by linear regression or penalized linear (SAM) regression. Panels A and B are histograms of the number of probe sets identified as dose-responsive using either a linear regression (blue bars) or a penalized linear regression in SAM (yellow bars) for deltamethrin and permethrin, respectively. Decile bins were defined by ranking probe sets by the mean raw expression summary score across all treatment conditions from lowest to highest and dividing the probe sets into equal groups (n = 3104 for deciles 2-8 and 3105 for deciles 1 and 10). Probe sets within each decile that were determined to be dose-responsive with each method were counted and plotted in the panels. Note that less probe sets were detected as significantly dose-responsive in the lower decile ranges using penalized regression while the number of probe sets identified as dose-responsive in the upper deciles was similar between the two methods.
Appendix B. Chapter 4 Supplementary Material
**Appendix B, Table 1. Taqman® qRT-PCR assay information.**

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*taken from Applied Biosystems web-site.
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Appendix B, Figure 1. Dimensional reduction of microarray data: effect of mean centering on measures of significance. Panels in the first column of the figure plot raw expression summary values for a probe set that has a significant interaction of time and treatment in a two-way ANOVA model (Nfkbia, top row), a probe set that has a significant main effect of treatment and no interaction of time and treatment (2610029k21, middle row) and a probe set with a significant main effect of time and no interaction of time and treatment (Ly86, bottom row). Significant effects observed with Nfkbia and 2610029k21 are exemplary of effects considering ‘interesting’ in the present study. Effects of time with no treatment are not of interest. Panels in the middle column are box plots of the expression summaries for each condition in the study before mean normalization for Nfkbia, 2610026K21 and Ly68. Significance values for analysis of the four groups with a One-Way ANOVA are listed above the panels. Note that all three of the genes are significant in the one-way ANOVA. Panels in the far right column are box plots of the expression summaries for each condition in the study after mean normalization. Note that the significant effect for Ly68 (bottom right corner panel) in the one-way ANOVA model is eliminated. Also, the two genes with treatment effects in the two-way ANOVA still have a significant model $p$-value in one-way ANOVA. Numbers in italicized type over each box plox are coefficients of variation of the data in each group. The C.V.s do not change following mean normalization. Mean normalized data was used in the SAFE enrichment analyses throughout the present study.