Drosophila melanogaster as a model to study insecticide mode of action

A thesis submitted to the University of Manchester for the degree of Doctor of Philosophy in the Faculty of Life Sciences

2015

Francesca Cash
# Contents

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Title</th>
<th>Pages</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>Contents</strong></td>
<td>2</td>
</tr>
<tr>
<td></td>
<td><strong>List of Figures</strong></td>
<td>6</td>
</tr>
<tr>
<td></td>
<td><strong>List of Tables</strong></td>
<td>9</td>
</tr>
<tr>
<td></td>
<td><strong>Abbreviations</strong></td>
<td>11</td>
</tr>
<tr>
<td></td>
<td><strong>Abstract</strong></td>
<td>14</td>
</tr>
<tr>
<td></td>
<td><strong>Declaration</strong></td>
<td>15</td>
</tr>
<tr>
<td></td>
<td><strong>Copyright Statement</strong></td>
<td>16</td>
</tr>
<tr>
<td></td>
<td><strong>Acknowledgements</strong></td>
<td>17</td>
</tr>
<tr>
<td></td>
<td><strong>Chapter 1: General Introduction</strong></td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>1.1 Types of synapse</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>1.1.1 ACh synthesis and synaptic vesicle loading</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>1.1.2 The synaptic vesicle cycle</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>1.1.3 Receptors and ACh recycling</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>1.2 Insecticides</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>1.3 <em>Drosophila</em> nervous system</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>1.4 Outline of thesis</td>
<td>47</td>
</tr>
<tr>
<td></td>
<td><strong>Chapter 2: Materials and Methods</strong></td>
<td>48</td>
</tr>
<tr>
<td></td>
<td>2.1 Fly maintenance</td>
<td>48</td>
</tr>
<tr>
<td></td>
<td>2.2 Fly stocks</td>
<td>48</td>
</tr>
<tr>
<td></td>
<td>2.3 Mortality assays</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>2.4 Tracking assays</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td>2.5 FACS and PCR</td>
<td>53</td>
</tr>
<tr>
<td></td>
<td>2.6 Creation of VACHT(^{1(M)}) and VACHT(^{G342R}) UAS lines</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>2.7 Electron microscopy</td>
<td>57</td>
</tr>
</tbody>
</table>
Chapter 3: Investigating Mode of Action of Spiroindolines in *Drosophila* Larvae

3.1 Introduction

3.2 Results

3.2.1 CASPP induced mortality in Drosophila larvae

3.2.2 VACht dependence of larval locomotion

3.2.3 Motoneuron identification and validation of mini analysis

3.2.4 The effect of CASPP on quantal release of ACh at cholinergic synapses

3.2.5 Alternative methods to reduce VACht activity

3.2.6 The effect of manipulating the VACht on spontaneous rhythmic currents

3.2.7 Altering levels of active VACht does not affect the postsynaptic response to ACh

3.2.8 Quantification of the overexpression of VACht in cha>VACht^{(+SYN)}

3.2.9 Expression of nAChR subunits in the aCC motoneuron

3.3 Discussion

3.3.1 Blocking the VACht causes mortality by reducing probability of SV release

3.3.2 Blocking VACht results in decreased frequency of SRCs

3.3.3 nAChR subunits Dα2, Dα5 and Dα6 are the most prevalent subunits in aCC

3.4 Conclusions

Chapter 4: The Effect of Overexpressing VACht on Synaptic Vesicle-Mediated Release

4.1 Introduction

4.2 Results

4.2.1 Mini amplitude, but not frequency, varies when VACht is overexpressed
4.2.2 Excitability of the postsynaptic motoneuron remains unchanged

4.2.3 Investigating vesicle number

4.2.3.1 Electron microscopy to determine effects to SV number

4.2.3.2 Number of loaded SVs remains unchanged when VACHT is overexpressed

4.2.3.3 Shibire and channelrhodopsin

4.2.4 Overexpressing VACHT causes an increased probability of release

4.3 Discussion

4.3.1 Overexpressing the VACHT causes an increase in mini frequency and inconsistently an increase in mini amplitude

4.3.2 There is no change in excitability of the postsynaptic cell when VACHT is overexpressed

4.3.3 Overexpressing VACHT does not increase SV number or volume

4.3.4 Overexpressing VACHT increases the probability of vesicle fusion

4.4 Conclusions

Chapter 5: The Giant Fiber System as a Model to Study Insecticide Mode of Action

5.1 Introduction

5.2 Results

5.2.1 The cholinergic synapse

5.2.1.1 VACHT

5.2.1.2 nAChR

5.2.2 VGSC

5.2.3 NMJ

5.2.4 GABA-gated chloride channel

5.2.5 Thoracic stimulation to bypass the chemical synapse

5.2.6 Compound injection

5.3 Discussion

5.4 Conclusions

Chapter 6: General Discussion

6.1 Conclusions

6.2 Outlook
References

Final word count: 54,438
## List of Figures

| Figure 1.1 | Schematic representation of the GAL4-UAS system | 19 |
| Figure 1.2 | Schematic representation of chemical and electrical synapses | 21 |
| Figure 1.3 | VACHT | 26 |
| Figure 1.4 | The SV cycle | 29 |
| Figure 1.5 | Insect nAChR and subunit relationships | 32 |
| Figure 1.6 | Drosophila CNS in third-instar larvae and locomotive neural pathway | 41 |
| Figure 1.7 | Schematic representation of aCC and RP2 motoneurons and their axonal projections to the muscle | 42 |
| Figure 1.8 | Synaptic inputs to aCC/RP2 motoneurons recorded in voltage clamp | 44 |
| Figure 1.9 | Drosophila adult CNS and GFS | 45 |
| Figure 2.1 | Structures of compounds used in this study | 52 |
| Figure 2.2 | Schematic representing primer binding positions of primers A-D within the VACHT sequence | 56 |
| Figure 2.3 | Schematic of third-instar CNS illustrating location of motoneurons used in electrophysiological recordings. | 60 |
| Figure 2.4 | Position of TTM and DLM muscles in the adult fly | 63 |
| Figure 3.1 | Insecticide targets within the cholinergic synapse | 66 |
| Figure 3.2 | CASPP compounds do not differ in potency and mortality is reduced when wild-type or resistant VACHT is overexpressed | 69 |
| Figure 3.3 | Crawling speed and distance travelled is decreased in CASPP treated larvae | 71 |
| Figure 3.4 | Inclusion of dye in the recording pipette does not affect mini recordings and analysis is not affected by choice of software | 73 |
| Figure 3.5 | Representative traces of mini recordings | 76 |
| Figure 3.6 | Amplitude and frequency of minis in different genotypes acutely fed CASPP | 77 |
| Figure 3.7 | Mortality in larvae fed with L(-)-vesamicol and the effect on minis of treatments aimed to replicate CASPP | 80 |
| Figure 3.8 | Representative SRC traces and analysis | 83 |
| Figure 3.9 | Manipulating the VACHT does not affect postsynaptic response to ACh | 85 |
| Figure 3.10 | VACHT transcript is increased in larvae which overexpress | 87 |
VAChT

Figure 3.11  Control genes in FACS sorted cells

Figure 3.12  Expression of nAChR subunits in aCC cells

Figure 4.1  Presence of cholinergic neurons and synaptic markers in third-instar Drosophila CNS

Figure 4.2  UAS_{VAChT^{+\text{SYN}}\text{SYN}} lacks 3 base pairs which correspond to a missing glutamine (Q) amino acid in the translated sequence

Figure 4.3  Effect of overexpressing VAChT on mini amplitude and frequency

Figure 4.4  Membrane excitability is not changed following overexpression of VAChT^{+\text{SYN}}. aCC motoneuron response to injected current in 4 pA steps and resting membrane potential in cha>VACHT^{+\text{SYN}} larvae

Figure 4.5  Two fixation protocols gave different clarity of images

Figure 4.6  Micrographs showing synapses in control CNS

Figure 4.7  Micrographs showing synapses in CASPP treated CNS

Figure 4.8  Micrographs showing synapses in cha>VACHT^{+\text{SYN}}

Figure 4.9  Micrographs illustrating range of synapse structures observed and analysis of synapse morphology

Figure 4.10  Example micrographs and analysis of active zone density

Figure 4.11  Altering VAChT does not affect synapse structure

Figure 4.12  Bafilomycin causes a rundown of SRCs

Figure 4.13  Number of ACh containing vesicles is unchanged in cha>VACHT^{+\text{SYN}} larvae and decreased in CASPP treated larvae

Figure 4.14  DMSO causes an increase in amplitude and/or duration of SRCs

Figure 4.15  SRC recordings are sensitive to external temperature

Figure 4.16  Channelrhodopsin can be used to evoke presynaptic cholinergic release

Figure 4.17  SRC duration is influenced by temperature in wild-type

Figure 4.18  CASPP treatment is able to increase rundown in amplitude of events

Figure 4.19  Events at 28°C are similar to events in CASPP treated larvae at room temperature (20-22°C)

Figure 4.20  aCC motoneurons in cha>ChR2^{H134R} respond to light pulses in the presence of synaptic signalling inhibitors
Figure 4.21  VACH{T}^{G342R} does not increase mini frequency

Figure 5.1  The *Drosophila* GFS

Figure 5.2  Mortality assays in adult *Drosophila*

Figure 5.3  EJPs in the DLM and TTM

Figure 5.4  DLM and TTM EJPs after compound treatment targeted at VACH{T}

Figure 5.5  DLM and TTM EJPs in flies that overexpress VACH{T} in cholinergic neurons

Figure 5.6  DLM and TTM EJPs in nAChR subunit null flies and after nAChR targeted compound treatment

Figure 5.7  DLM and TTM EJPs after compound treatment targeted at VGSC

Figure 5.8  DLM and TTM EJPs after compound treatment targeted at glutamatergic NMJ

Figure 5.9  DLM and TTM EJPs after compound treatment targeted at GABA-gated chloride channels

Figure 5.10  DLM and TTM EJPs during thoracic stimulation

Figure 5.11  DLM and TTM EJP following frequency after injection of compounds that target nAChR

Figure 5.12  DLM and TTM EJP following frequency after injection of CASPP and deltamethrin

Figure 6.1  Model of single transporter per SV vs. multiple transporters

Figure 6.2  Steady state and set point models of SV loading
List of Tables

Table 1.1  Neurotoxic insecticide classes and modes of action  37
Table 2.1  Fly stocks used in this study  49
Table 2.2  Compounds used in contact/feeding assays and GFS recordings  51
Table 2.3  Primer sequences  54
Table 3.1  Overexpressing VACHT reduces sensitivity to CASPP  70
Table 3.2  Hierarchy of expression of nAChR subunits in whole CNS and FACs sorted aCC cells of RRa-GFP larvae  90
Table 4.1  Summary of electron microscopy analysis  108
Table 5.1  Overview of change in DLM and TTM EJPs after compound treatment targeted at VACHT  141
Table 5.2  Overview of change in DLM and TTM EJPs after compound treatment targeted at nAChR  148
Table 5.3  Overview of change in DLM and TTM EJPs after compound treatment targeted at VGSC  151
Table 5.4  Overview of change in DLM and TTM EJPs after compound treatment targeted at the NMJ  155
Table 5.5  Overview of change in DLM and TTM EJPs after compound treatment targeted at the GABA-gated chloride channel  157
Table 5.6  Mean values of response latency, amplitude and following frequency in DLM and TTM muscles after compound treatment targeted at VACHT  170
Table 5.7  Mean values of response latency, amplitude and following frequency in DLM and TTM muscles after compound treatment targeted at nAChR  171
Table 5.8  Mean values of response latency, amplitude and following frequency in DLM and TTM muscles after compound treatment targeted at the VGSC  172
Table 5.9  Mean values of response latency, amplitude and following frequency in DLM and TTM muscles after compound treatment targeted at the NMJ  173
Table 5.10 Mean values of response latency, amplitude and following frequency in DLM and TTM muscles after compound treatment targeted at the GABA-gated chloride channel  173
Table 5.11  Mean values of response latency, amplitude and following frequency in DLM and TTM during head and thoracic stimulation
Abbreviations

acetyl-CoA  acetyl coenzyme A
ACh  acetylcholine
AChE  acetylcholine esterase
ADNFLE  autosomal dominant nocturnal frontal lobe epilepsy
AEL  after egg laying
ATP  adenosine triphosphate
cAMP  cyclic 3',5'-adenosine monophosphate
CASPP  5Cl-CASPP
cDNA  complementary DNA
ch  cha<sup>B19</sup>-GAL4
cha<sup>B19</sup>-ChR2<sup>H134R</sup>  cha<sup>B19</sup>-GAL4>UAS_ChR2<sup>H134R</sup>
cha<sup>B19</sup>-VAcChT<sup>RNAi</sup>  cha<sup>B19</sup>-GAL4>UAS_VAcChT<sup>RNAi</sup>
cha<sup>B19</sup>-VAcChT<sup>(M)</sup>  cha<sup>B19</sup>-GAL4>UAS_VAcChT<sup>(M)</sup>
cha<sup>B19</sup>-VAcChT<sup>(SYN)</sup>  cha<sup>B19</sup>-GAL4>UAS_VAcChT<sup>(SYN)</sup>
cha<sup>B19</sup>-VAcChT<sup>G342R</sup>  cha<sup>B19</sup>-GAL4>UAS_VAcChT<sup>G342R</sup>
cha<sup>B19</sup>-VAcChT<sup>Y49N</sup>  cha<sup>B19</sup>-GAL4>UAS_VAcChT<sup>Y49N</sup>
ChAT  choline acetyltransferase
ChR2  channelrhodopsin<sup>2H134R</sup>
CNS  central nervous system
CPG  central pattern generator
CS  CantonS
DCJW  decarbomethoxylated JW062
Ddc  dopa decarboxylase
DDT  dichlorodiphenyltrichloroethane
DLM  dorsal longitudinal muscle
DLMn  dorsal longitudinal motoneuron
DMSO  dimethyl sulfoxide
DNA  deoxyribonucleic acid
DVM  dorsoventral muscle
EJP  excitatory junctional potentials
EPP  endplate potential
FACS  fluorescence activated cell sorting
FF  following frequency
GABA  γ-aminobutyric acid
GCI  giant commissural interneuron
GF   giant fiber
GFP  green fluorescent protein
GFS  giant fiber system
GluR glutamate gated ion channel
ISN  intersegmental
LD100 lethal dose that causes 100% mortality
LD50 lethal dose that causes 50% mortality
LGIC ligand gated ion channel
mAChR muscarinic acetylcholine receptors
mEJP miniature excitatory junctional potential
mEPC miniature endplate currents
mEPSC miniature excitatory postsynaptic currents
mRNA messenger RNA
nAChR nicotinic acetylcholine receptors
NMAD N-methyl-D-aspartate
NMJ neuromuscular junction
NSF N-ethylmaleimide-sensitive fusion protein
OrR OregonR
PCR polymerase chain reaction
PSI periphery synapsing interneuron
qRT-PCR quantitative real-time PCR
RDL resistance to dieldrin
RL response latency
RNA ribonucleic acid
RNAi RNA interference
RRa RRa-GAL4
RRa>nAChR_Dα7 RRa-GAL4>UAS_nAChR_Dα7.GFP
RRa-GFP RRa-GAL4:CD8GFP
SEM Standard error of the mean
shi²⁶² shibire⁶²⁶²
SN segmental
SNAP synaptosomal-Associated Protein
SNARE soluble NSF attachment receptor
SRC spontaneous rhythmic current
SV synaptic vesicle
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>TM</td>
<td>transmembrane</td>
</tr>
<tr>
<td>TN</td>
<td>transverse</td>
</tr>
<tr>
<td>TTM</td>
<td>tergotrochanteral muscle</td>
</tr>
<tr>
<td>TTMn</td>
<td>tergotrochanteral motor neuron</td>
</tr>
<tr>
<td>TTX</td>
<td>tetrodotoxin</td>
</tr>
<tr>
<td>UAS</td>
<td>upstream activator sequence</td>
</tr>
<tr>
<td>VACHT</td>
<td>vesicular acetylcholine transporter</td>
</tr>
<tr>
<td>VACHT(M)</td>
<td>UAS_VACHT transcript made at the University of Manchester</td>
</tr>
<tr>
<td>VACHT(SYN)</td>
<td>UAS_VACHT transcript obtained from Syngenta</td>
</tr>
<tr>
<td>VGAT</td>
<td>vesicular GABA transporter</td>
</tr>
<tr>
<td>VGKC</td>
<td>voltage gated potassium channel</td>
</tr>
<tr>
<td>VGLUT</td>
<td>vesicular glutamate transporter</td>
</tr>
<tr>
<td>VGSC</td>
<td>voltage gated sodium channel</td>
</tr>
<tr>
<td>VIAAT</td>
<td>vesicular inhibitory amino acid transporter</td>
</tr>
<tr>
<td>VMAT</td>
<td>vesicular monoamine transporter</td>
</tr>
<tr>
<td>VNC</td>
<td>ventral nerve cord</td>
</tr>
</tbody>
</table>
Abstract
The University of Manchester
Francesca Cash
Submitted for the degree of Doctor of Philosophy
Drosophila melanogaster as a model to study insecticide mode of action
11/09/2015

The development of novel insecticides requires insect models to determine mode of action. This project aimed to assess Drosophila as a model system to study insecticide mode of action. Drosophila larvae were used to confirm the action of the spiroindoline insecticide 5CI-CASPP (CASPP). Experiments presented confirm that this compound inhibits acetylcholine (ACh) loading into synaptic vesicles (SVs) by inhibiting the vesicular acetylcholine transporter (VACHT). Mortality assays showed that CASPP induced lethality is reduced by either overexpression of VACHT or expression of a resistant transporter (VACHT\textsuperscript{Y49N}). Larval tracking demonstrated a sub-lethal effect of CASPP on cholinergic-regulated locomotion. Whole-cell patch recordings from identified motoneurons, that receive excitatory cholinergic synaptic input, showed reduced frequency of release of ACh-containing SVs (i.e. minis) following exposure to CASPP. Mini amplitude was unaffected. By contrast, overexpression of VACHT resulted in a marked increase in frequency of minis but again no change to amplitude. Expression of VACHT\textsuperscript{G342R} that, in C.elegans, is unable to bind synaptobrevin does not affect either mini frequency or amplitude. This suggests that VACHT may also govern ACh release, in addition to SV loading.

While overexpression of wild-type VACHT increases mini frequency and not amplitude, increases to both were observed following expression of a VACHT variant with a polymorphism (missing glutamine; Q) in a unique polyQ domain. Ultrastructural examination of the active zone found that changes to SV release were not accompanied by changes to active zone morphology, SV size or number. This was supported by the finding of no significant difference in total SVs released in the –Q VACHT variant when SV recycling was blocked. Taken together, the data are indicative that individual SVs are filled to a greater level following expression of this VACHT variant. This identifies the polyQ domain as a potential regulator of SV loading and, moreover, may provide an attractive target for novel insecticide development.

This project also assessed the Giant Fiber System (GFS) in adult Drosophila as a potential medium throughput model to rapidly identify insecticide mode of action. The GFS is activated by electrical stimulation and output measured as excitatory junctional potentials (EJPs) in leg and flight muscles. Flies were treated with compounds of different known modes of action (cholinergic, glutamatergic etc.) in order to characterise effect. Novel compounds with unknown modes of action were also tested and effects compared. This system is suitable to indicate insecticides with a cholinergic mode of action but requires additional characterisation to fully understand the relationship between mode of action of compounds and GFS output.
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Chapter 1

General Introduction

The control of pest insect species has been a challenge since humans have been cultivating crops. The first recorded use of an insecticide was sulphur in 2000BC (Fishel, 2009). Since that time insecticides have progressed from those derived from plants, such as nicotine from the tobacco plant, to synthetic compounds. With the increased use of insecticides came the appearance of insecticide resistance in pest species (Casida and Quista d, 1998). An insecticide puts a selection pressure on the pest population, favouring individuals who are resistant. One tactic to prevent the occurrence of resistance is to rotate use of insecticides with different modes of action. The mode of action describes the mechanism by which the compound acts within the organism. Resistance and increasing concern for the impact of current insecticides on the environment means that the development of novel insecticides is crucial.

When a new compound is developed as a potential insecticide, its mode of action must be verified. This requires well characterised insect models whose response to an insecticidal compound can indicate mode of action. In this thesis I use *Drosophila melanogaster* larvae as a low throughput model to study the mode of action of a novel insecticide, 5CI-CASPP (CASPP) of the class spiroindolines, which have a target at the cholinergic synapse. This project also aims to evaluate a medium throughput adult assay as a tool to decipher insecticide mode of action. *Drosophila* as an insect model organism has many merits. The genome has been sequenced and annotated, revealing just over 14,000 genes located on 4 chromosomes (Pandey and Nichols, 2011). *Drosophila* has tractable genetics, simple behaviours and a relatively simple central nervous system (CNS) with fewer neurons compared to more complex animals. They also have neurons that can be easily identified by position, axonal and dendritic projections or by transgenic expression of green fluorescence protein (GFP) (Baines and Pym, 2006). A significant amount of information, including genetic tools and experimental techniques are available with easily accessible databases (FlyBase: http://flybase.org/) and stock centres (Bloomington stock centre: http://flystocks.bio.indiana.edu/) (Schneider, 2000). Genetic tools available include
site specific insertion of transgenes, RNAi strains and a GAL4-UAS system that allows expression of target genes in chosen tissues or cells (Duffy, 2002; Nichols, 2006; Dietzl et al., 2007; Pandey and Nichols, 2011). The GAL4-UAS system utilises a mechanism of gene regulation identified in yeast *Saccharomyces cerevisiae*. The GAL4 gene encodes a protein that binds to an upstream activating sequence (UAS), activating transcription of the UAS controlled gene. In *Drosophila*, one fly strain carries the GAL4 under control of a native gene promotor (specifies spacial and temporal expression of target gene) and a second fly strain carries the target gene under control of the UAS (Fig. 1.1). When the two strains are crossed, the offspring will express the target gene only where the GAL4 is expressed (Nichols, 2006). For example, when the cha-GAL4 driver line (GAL4 expression is under the control of the promotor for choline acetyltransferase) is crossed to a UAS controlled GFP line, the offspring will express GFP in cholinergic neurons only.

**Figure 1.1** Schematic representation of the GAL4-UAS system

The fly strain on the left expresses the yeast transcription factor GAL4 under the control of enhancer elements for a specific gene. The strain on the right contains a responder transgene (reporter gene or RNAi element, for example). When the two strains are crossed the offspring express the responder gene in tissues defined by the gene specific promoter. Adapted from Nichols (2006).

Compounds that change the way a protein functions not only have potential as insecticides, they also offer an opportunity to study the protein that they target. The novel spiroindoline class of insecticides bind to the vesicular acetylcholine transporter (VACHT) and inhibit its function (Sluder et al., 2012). The VACHT loads the neurotransmitter acetylcholine (ACh) into synaptic vesicles (SVs) at the
cholinergic presynapse (Usdin et al., 1995). This thesis also aims to further understanding of the function of the VACChT at a central cholinergic synapse using spiroindoline variant CASPP and transgenic overexpression to manipulate expression. *Drosophila* has another advantage here in that the nervous system shows many parallels to mammals, such as conservation of ion channels and signalling proteins. At the primary amino acid level, *Drosophila* synaptic proteins are >70% similar to their mammalian equivalents and there are very few mammalian proteins which don’t have a *Drosophila* ortholog (Littleton and Ganetzky, 2000; Littleton, 2000; Lloyd et al., 2000). Furthermore, the *Drosophila* VACChT shows high sequence identity with human and rat VACChT (49%) (Kitamoto et al., 1998). Therefore, understanding the *Drosophila* cholinergic synapse will also further understanding of the role of cholinergic signalling in humans, and specifically the VACChT. This project also aims to contribute to knowledge about a central cholinergic synapse in *Drosophila* larvae by elucidating the subunit composition of nicotinic acetylcholine receptors (nAChR). This will further improve this system as a model to study central cholinergic synapses.

This thesis focuses on the cholinergic synapse in *Drosophila* larvae to understand cholinergic function and CASPP mode of action, and a neuronal system in adult as a model to study insecticide mode of action. Therefore, this chapter will describe synaptic connections with focus on the cholinergic synapse and the enzymes and proteins involved. It will also discuss the role of insecticides and current problems facing their use as well as describing the novel spiroindoline insecticide class. Finally, this chapter will describe the *Drosophila* nervous system with specific focus on how it can be utilised to study insecticide mode of action and cholinergic function.

### 1.1 Types of synapse

Neurons must communicate in order for the nervous system to function. Signalling between neurons can be achieved through electrical or chemical synapses, or a mixture of both (Fig. 1.2). Although not relevant to this thesis, it is worth noting that there are also less direct ways in which neurons can influence other neurons: volume transmission by diffusion of neurotransmitter through the extracellular space to remote targets and electrical fields generated by, for example, the hippocampus that can influence the activity of nearby neurons (Jefferys, 1995; Fuxe et al., 2013).
Electrical synapses involve direct contact between neurons through gap junction channels. Chemical synapses operate by the release of a chemical signal (neurotransmitter) from one neuron that is detected by another (Pereda, 2014). Detection of neurotransmitter can be by ionotropic or metabotropic receptors. Ionotopic receptors are ligand gated ion channels (LGIC) that transmit cations or anions on binding of the neurotransmitter. Metabotropic receptors initiate second messenger cascade signals that can lead to excitatory or inhibitory effects (Marrs and Maynard, 2013). Electrical synapses do not require neurotransmitter, they consist of gap junctions that directly connect the interiors of two adjacent cells. This enables the passage of electrical currents and small molecules (calcium, cyclic AMP and inositol-1,4,5-trisphosphate) (Pereda, 2014). Gap junctions can be bidirectional or preferentially transmit depolarizing current in one direction (Marder, 1998; Phelan et al., 2008). The gap junction consist of two hexameric connexin ‘hemichannels’, one from each cell. In invertebrates the gap junction proteins are innexins. Unlike chemical synapses, electrical synapses cannot amplify or transform presynaptic signals but they are able to coordinate the activity of a group of interconnected neurons because of the instantaneous nature of their transmission (Pereda, 2014).

**Figure 1.2** Schematic representation of chemical and electrical synapses

A. At the chemical synapse SVs containing neurotransmitter fuse with the presynaptic nerve terminal membrane, releasing neurotransmitter into the synaptic cleft. At the postsynaptic site, neurotransmitter binds to ionotropic and/or metabotropic receptors. B. At the electrical synapse two neurons are directly coupled by gap junction channels, allowing exchange of electrical current and small molecules. Adapted from Pereda et al (2014).
Chemical synapses can be excitatory (e.g. glutamate and ACh), inhibitory (e.g. GABA) or modulatory (e.g. serotonin and octopamine). At the cholinergic synapse acetylcholine (ACh) is the neurotransmitter. ACh and glutamate are the two main excitatory neurotransmitters (Marrs and Maynard, 2013). In mammals glutamate is the predominant excitatory neurotransmitter in the central nervous system (CNS) and ACh plays this role at the neuromuscular junction (NMJ). However, cholinergic synapses are still present throughout the mammalian brain (Dineley et al., 2014). In Drosophila the reverse is true and ACh is the main excitatory neurotransmitter in the CNS, and glutamate at the NMJ. The majority of the CNS is cholinergic in Drosophila larvae and the adult CNS contains many cholinergic neurons in the optic lobes, antennal lobes, mushroom body calyx and some areas of the subesophageal ganglion and throughout the thoracic-abdominal centre (Gorczyca and Hall, 1987; Yasayama and Salvaterra, 1999; Salvaterra and Kitamoto, 2001; Kim et al., 2011). In humans, cholinergic neurons are found in the basal forebrain, striatum, cerebral cortex, mesopontine tegmental nuclei, cranial motor nuclei and spinal motoneurons (Oda and Nakanishi, 2000). Above the brain stem there are three major subsystems that innervate almost every neural area. 1, the pedunculopontine tegmentum and laterodorsal pontine tegmentum (found in the mesopontine tegmental nuclei) innervate the thalamus and midbrain dopaminergic areas and also have innervations descending into the caudal pons and brain stem. 2, the basal forebrain that has broad projections throughout the cortex and hippocampus and 3, cholinergic interneurons in the striatum that have rich local innervation throughout the striatum and olfactory tubercle (Dani and Bertrand, 2007). The cholinergic system plays a role in learning, memory, attention and synaptic plasticity (Dani and Bertrand, 2007; Deianna et al., 2011; Prado et al., 2013; Yakel, 2013). Deficiencies in this system are linked to diseases such as Alzheimer’s, schizophrenia and epilepsy. Alzheimer’s sufferers show a loss of cholinergic neurons in the basal forebrain and Parkinson’s sufferers show progressive loss of cholinergic neurons in the basal forebrain and decrease of cholinergic activity in the hippocampus (Auld et al., 2002; Terry and Buccafusco, 2003; Dani and Bertrand, 2007; Schliebs and Arendt, 2011; Hall et al., 2014). In epilepsy there is some evidence that altered cholinergic modulation initiates seizure events (Zimmerman et al., 2008).

At the cholinergic presynapse ACh is synthesised from acetyl coenzyme A (acetyl-CoA) and choline, catalysed by choline acetyltransferase (ChAT). ACh is loaded into SVs via the VACHT. SVs are formed by budding from presynaptic endosomes at nerve terminals. PC12 cells transfected with tagged vesicle-associated
membrane protein (VAMP) show localisation of this protein to endosome and in small vesicles resembling SVs (Lichtenstein et al., 1998). Endosome membrane curvature is induced by endophilin and it is likely that dynamin plays an important role in cleavage of the SVs (Jähne et al., 2015). The proteins found in the membrane of SVs are synthesised in the cell body and transported to the nerve terminal for inclusion in SVs (Rizzoli, 2014). Loaded SVs fuse with the plasma membrane at the nerve terminal releasing ACh into the synaptic cleft. Detection of ACh can be by nicotinic or muscarinic acetylcholine receptors (nAChR/mAChR). Remaining ACh in the synaptic cleft is hydrolysed by acetylcholine esterase (AChE) to choline and acetate. Choline is transported back into the presynaptic terminal for recycling (Fournier et al., 1987; Silman and Sussman, 2005). The components of this cycle are discussed in more detail below.

### 1.1.1 ACh synthesis and synaptic vesicle loading

The enzyme ChAT is responsible for ACh synthesis and is found in the cytoplasm of presynaptic cholinergic neurons. This soluble enzyme catalyses ACh synthesis by transferring an acetyl group from acetyl-CoA to choline, producing ACh. Acetyl-CoA is produced from pyruvate made from glucose in the mitochondria and choline is transported into the neuron via the Na⁺ dependent high affinity choline transporter. Approximately half the choline comes from recycled ACh, choline can also be made by the breakdown of phosphatidylcholine (Oda, 1999; Taylor and Brown, 1999). ChAT controls the availability of ACh, and levels of ChAT and ACh were found to be correlated in Drosophila heads (Salvaterra and McCaman, 1985). ChAT is synthesised in the soma and transported to nerve terminals, it is primarily cytoplasmic but there is also a membrane bound form that is associated with plasma membranes and SV membranes (Taylor and Brown, 1999; Sha et al., 2004). In the adult Drosophila head both soluble and membrane bound forms of the enzyme are found, but the membrane bound form does not appear to be associated with SVs (Pahud et al., 1998). The activity and distribution of ChAT can be modified by phosphorylation of the enzyme (Dobranksy, 2005). The ChAT gene shares a phylogenetically conserved cholinergic locus with VACHT (Alfonso et al., 1994, Usdin et al., 1995; Mallet et al., 1998). In Drosophila VACHT shares a common first exon with ChAT and the remainder of the single coding exon for VACHT is within the first intron of ChAT. Production of two distinct mRNAs in Drosophila is believed to
be by alternative RNA processing from a common primary transcript (Kitamoto et al., 1998). Synthesised ACh is transported into SVs where it is stored at concentrations much higher than in the cytoplasm. Therefore, ACh is transported against its concentration gradient. This is achieved by exchange of ACh with 2 protons within the vesicle (Prado et al., 2013). Protons are transported into the SV by the V-ATPase proton pump.

The V-ATPase proton pump acidifies the SV lumen, a necessary step for ACh loading (Drory and Nelson, 2006; Poëa-Guyon et al., 2013; Vavassori and Mayer, 2014). This sets up the electrochemical potential required for loading of neurotransmitter. The proton pump is the largest component of the SV (1000kDa), therefore only one or two are usually present per SV (van der Kloot, 2003). The proton pump consists of two sub-complexes: V₀, the membrane complex that mediates proton translocation and V₁, the larger peripheral complex that carries the ATPase activity (Südhof, 2004; Vavassori and Mayer, 2014). V₁ is cytosolic and is made up of 8 subunits (A-H). It is subunit A that is responsible for catalysing ATP hydrolysis. V₀ is made up of 9 subunits and may also act as a pH sensor (Poëa-Guyon et al., 2013). There is also some evidence for a role in SV fusion for the V₀ complex, possibly by regulation of the SNARE (soluble NSF attachment receptor) complex assembly (Hiesinger et al., 2005; Vavassori and Mayer, 2014). Once the SV contains protons it is able to exchange these for ACh molecules via the VACHT.

The VACHT loads ACh into SVs and is essential for cholinergic function (Usdin et al., 1995). VACHT knockout mice do not survive more than a few minutes after birth, Drosophila homozygous null mutants do not survive the larval stage and C.elegans mutants are uncoordinated (Kitamoto, 2000; Alfonso et al., 1993; de Castro et al., 2009). The VACHT is found at the cholinergic presynapse so is present in the central and peripheral nervous systems in mammals and Drosophila. VACHT is a member of the vesicular class of neurotransmitter transporters (Martin and Krantz, 2014). Other transporters include those for glutamate (VGLUT), monoamines (VMAT) and GABA and glycine (VGAT and VIAAT) (Fei and Krantz, 2009). VACHT was first discovered in the electric organ of the Torpedo and in C.elegans (Alfonso et al., 1993; Roghani et al., 1994). In humans the genes encoding ChAT and VACHT are found on chromosome 10, and are believed to be transcribed in the same direction. In Drosophila melanogaster the gene locus is found at chromosome 3R. In some diseases that are characterised by decreased cholinergic tone, a decrease in VACHT is found. More specifically, VACHT levels have been shown to decrease in diseases including Alzheimer’s and Huntingdon’s (Chen et al., 2011;
Efange et al., 1997; Smith et al., 2006). VACHT knockdown mice have impaired performance in cognitive tasks and social recognition that can be linked to poor memory (Prado et al., 2006). VACHT may also be important in the peripheral immune response and VACHT knockdown mice have an increased immune response when injected with parasites (Ribeiro et al., 2012).

A predicted sequence of VACHT suggests a protein with 12 transmembrane domains, hydrophilic amino and carboxyl termini and a large glycosylated intravesicular loop between domains 1 and 2 (Usdin et al., 1995; Arvidsson et al., 1997; Fei and Krantz, 2009). It is proposed that the 12 domains fold into 2 bundles containing transmembrane helices I-VI and VII-XII with the N- and C- terminals on the cytoplasmic side. This folding forms a central transport path that allows the binding domains access to either the interior of the vesicle or to the cytoplasm, via a rocking motion. The ACh binding site is proposed to be close to tryptophan331 at the beginning of transmembrane helix VIII in the luminal part of the transport channel and aspartate398 may be involved in translocation of one of the protons (Prado et al., 2013). The VACHT is of the family which also includes VMAT. The proteins are similar and both rely on an electrical gradient for filling. Cytoplasmic ACh is 1-4mM and final concentration of ACh in SVs is 160-600mM depending on species (Prado et al., 2013). VACHT concentrates ACh 100-fold in the SV in vivo, 30-fold less than predicted from the available free energy produced by exchange of two protons (Parsons, 2000). The VACHT exchanges two protons for each molecule of ACh, at a rate of 1/s and thousands of ACh molecules are required to form a quantum (Fig. 1.3) (Varoqui and Erickson, 1996; Nguyen et al., 1998; Chaudhry et al., 2008; Prado et al., 2013). The discrepancy between predicted and actual concentration of ACh and the fact that VACHT is a slow transporter suggests that ACh storage is somehow regulated and that the VACHT is a rate limiting step (Parsons, 2000; Prado, 2013). The number of transporters per SV is unknown, but has been estimated to be between one and three but up to 10 at central synapses (Van der Kloot, 2003; Takamori et al., 2006). Surprisingly in VACHT knockout mice, small amplitude quantal release can be detected at low frequency, suggesting some diffusion and accumulation of ACh in vesicles without the presence of VACHT (de Castro et al., 2009).
A link between amount of neurotransmitter stored within a single vesicle (quantal size) and expression of neurotransmitter transporters has been reported for the VAChT, VGLUT and VMATs (Song et al., 1997; Lima et al., 2010; Rodrigues et al., 2013; Daniels et al., 2004; Wilson et al., 2005; Colliver et al., 2000). A change in frequency of vesicle release (quantal release frequency) has also been described for the VAChT and VGLUT (Song et al., 1997; Parsons et al., 1999; Daniels et al., 2006; Lima et al., 2010; Rodrigues et al., 2013). A VAChT knockdown model in mice results in decreased amplitude and frequency of quantal ACh release (Prado et al., 2006; Lima et al., 2010; Rodrigues et al., 2013). SVs were also smaller and showed altered distribution near the active zone, however total SV number was unchanged (Rodrigues et al., 2013). Interestingly in control mice when VAChT was blocked by vesamicol SVs were smaller but did not show altered distribution. When vesamicol was used to block VAChT at frog NMJ, miniature endplate currents (mEPCs; representative of quantal SV release) were decreased in amplitude, accompanied by decreased SV size (Van der Kloot, 2000). A smaller population of mEPCs was also found in rat motor endplate treated with vesamicol (Searl et al., 1991). Vesamicol application at the twitch muscle fiber of garter snake caused decreased frequency of mEPCs but did not affect amplitude (Parsons et al., 1999). VAChT overexpression at developing Xenopus spinal neurons caused increased frequency and amplitude of mEPCs but did not affect amplitude (Parsons et al., 1999). In Drosophila, two loss of function VAChT lines (VAChT¹ and VAChT²) are recessive lethal; VAChT¹ causes death in the embryo and VAChT² is lethal at the larval stage. Heterozygous adults showed defects at a cholinergic synapse under electrical stimulation, suggestive of a decrease in ACh release from nerve terminals (Kitamoto et al., 2000). VGLUT has been shown to determine glutamate load in isolated SVs.

Figure 1.3 VAChT

The VAChT loads ACh into SVs by exchange of two protons from the interior of the SV. The proton pump responsible for creating the proton gradient is also shown. Adapted from Chaudhry et al (2008).
from rat cerebral cortex. Overexpression of VGLUT in cultured hippocampal neurons caused increased amplitude of quantal release, measured as miniature excitatory postsynaptic currents (mEPSCs), frequency was not affected (Wilson et al., 2005). At Drosophila NMJ increased VGLUT expression caused increased quantal size, reflected as increased amplitude of miniature excitatory junctional potentials (mEJPs) and increased size of SVs, and a small increase to frequency of mEJPs. Decreased VGLUT caused decreased frequency of mEJPs with no change to amplitude. SVs were smaller on average and total number was decreased (Daniels et al., 2004, 2006). These conflicting reports make it difficult to predict how inhibition of VAChT by CASPP or overexpression of VAChT would affect quantal release at a Drosophila cholinergic synapse. However, overall the literature suggests that decreased transporter expression or block leads to decreased frequency of postsynaptic currents with varied effect on amplitude and overexpression leads to increased amplitude and frequency. The main role of the VAChT is to load ACh into vesicles but there is increasing evidence that the VAChT interacts with other proteins to perform other roles associated with the SV cycle. A study using synaptosomes extracted from porcine brains found a potential structural and functional coupling between ACh synthesis and ACh loading into vesicles (Sha et al., 2004). There is also evidence to suggest that the C-terminal tail of VAChT interacts with clathrin adaptor proteins that are involved in endocytosis (Barbosa et al., 2002). An interaction between synaptobrevin and VAChT has been shown in C.elegans and may suggest a role for VAChT in SV release (Sandoval et al., 2006). Synaptobrevin is involved in SV docking and fusion (Südhof, 2013). Manipulation of VAChT pharmacologically or genetically may effect SV release through this or an unknown interaction. I have therefore included a description of SV release in this introduction.

1.1.2 The synaptic vesicle cycle

Depolarisation of the nerve terminal by an action potential activates voltage gated calcium channels, leading to an influx of calcium. This influx stimulates large numbers of SVs to undergo exocytosis. SVs can also undergo exocytosis in a more poorly understood spontaneous manner, this usually involves singular SV fusion (Südhof, 2012; Pang et al., 2011). The journey from loaded vesicle to exocytosis is a complex one that is still not fully understood. For exocytosis to be achieved the SV
must become very close to an area of the presynaptic plasma membrane known as the active zone where the SV becomes ‘docked’. RIM and RIM-BP proteins organise the active zone (including recruitment of calcium channels to release sites) and orchestrate the SV attachment site in conjunction with Munc13, RAB3 and RAB27. RAB3 and RAB27 are located on the SV and RIMS are essential for normal SV docking. The SNARE proteins are required to further bring the membranes of the SV and nerve terminal into close proximity. SNARE proteins include synaptobrevin located on SVs, syntaxin1 and SNAP-25 (synaptosomal-Associated Protein-25) found on the presynaptic plasma membrane. Parts of the SNARE proteins, termed SNARE motifs interact to form the SNARE complex. The R-SNARE motif is within synaptobrevin, Qa-SNARE of syntaxin 1 and Qb and Qc SNARE motifs of SNAP-25. The four SNARE motifs twist into a four helical SNARE complex bundle. This causes the SV to be pulled close to the plasma membrane, an action termed ‘zippering’, here the SNARE complex is in the trans-SNARE formation, after fusion pore opening the complex changes to cis-SNARE when bound by NSF (N-ethylmaleimide-sensitive fusion protein) and SNAP (Fig. 1.4) (Südhof, 2013). SVs are classed as ‘docked’ before the SNARE complex has formed, and ‘primed’ for fusion after SNARE complex assembly (Südhof, 2004). The SM (sec1/Munc18) proteins and Munc13 also play an important role in SNARE complex formation. Synaptotagmins and complexin govern fast, calcium dependent exocytosis. Synaptotagmin, located on the SV has two cytoplasmic C2 domains that bind to phospholipids (of the plasma membrane) in the presence of calcium. Complexins are found in the cytoplasm and stabilize the partially zippered SNARE complex and sensitise it to activation by synaptotagmin. They may also play a role in blocking progression of the SNARE complex, until calcium is present (Jahn and Fasshauer, 2012; Südhof, 2013).
After fusion pore opening, the SVs are recycled (Richmond and Broadie, 2002). There are three pathways for SVs to recycle: 1) SVs are reacidified and reloaded with neurotransmitter without undocking, termed “kiss-and-stay”; 2) SVs undock and are recycled, termed “kiss-and-run” and 3) SVs undergo clathrin-mediated endocytosis and either recycle directly or after passing through an endosomal intermediate (Südhof, 2004; Südhof and Rizo, 2011). The first two recycling methods are fast and predominantly used to recycle vesicles to the readily releasable pool during low stimulation frequencies. The slower recycling is thought to act more at higher frequency stimulations. SVs involved in the exo- and endocytosis cycle are referred to as the recycling pool, this includes the readily releasable pool.
releasable pool. During intense stimulation the recycling pool is replenished by the reserve pool of SVs (Rizzoli and Bertz, 2005). It appears that in many systems the number of vesicles recycled on a regular basis is a very small proportion of the population. A study that observed SV recycling in a number of organisms found that only 1-5% of vesicles shows uptake of dye over several hours in NMJ of Drosophila, C.elegans, leg muscle of the locust, tail muscle in zebrafish, chest muscle in frog and ear elevating muscle in mouse. This was in the absence of stimulation (Denker et al., 2011). The same was also reported in the CNS of Drosophila. It therefore seems that the recycling pool is small with a larger reserve pool less frequently used (Denker et al., 2011).

Calcium dependent exocytosis of multiple SVs (evoked release) activate many postsynaptic receptors and are able to initiate action potentials, playing a clear role in neuronal transmission. Spontaneous single SV release can be calcium dependent or independent (Pang et al., 2011; Kavalali, 2015). Calcium independent release is considered to be caused by low probability conformational changes in the SV release machinery. Some proteins of the SV release machinery are essential for evoked but not spontaneous release and vice versa, suggesting different mechanisms for spontaneous and evoked release (Kavalali et al., 2011; Schneggenburger and Rosenmund, 2015). Spontaneous release is important in development and can initiate biochemical signalling that leads to maturation and stability of synaptic networks and can also cause local dendritic protein synthesis. Spontaneous SV release is believed to play a role in the development and homeostasis of synapses. In Drosophila, complexin null mutants that have increased spontaneous SV release, show overgrowth of synapses (Huntwork and Littleton, 2007; Choi et al., 2014). In cultured hippocampal neurons spontaneous glutamate release can regulate NMDA receptor expression (Lee et al., 2010).

1.1.3 Receptors and ACh recycling

ACh released from SVs is detected by receptors that can be located on the pre- and postsynaptic membranes. The nAChR is a large glycoprotein complex of ~ 290kDa (Miyazawa et al., 1999) and is a member of the family of Cys-loop LGICs. All members of this family have a pair of disulphide-bonded cysteines separated by 13 residues in common. Members of this family include: 5-HT3, GABA_A, GABA_C and glycine receptors (Corringer et al., 2000). Studies in Drosophila and C. elegans
show that invertebrates have additional ligand gated ion channels LGIC: GABA-gated cation channels, 5-HT$_3$-gated chloride channels, glutamate gated chloride channels and histamine gated chloride channels (Littleton and Ganetzky, 2000).

The nAChRs are selective for cations (excluding one anion selective invertebrate channel) and are usually non-rectifying (Karlin, 2002). In the pre-synaptic membrane they are involved in neuromodulation. Post-synaptically they are able to initiate action potentials by depolarisation of the membrane when bound by ACh. The ion channel is also sensitive to other compounds, including nicotine, hence its name. In vertebrates the neuronal nAChRs influence the release of dopamine, glutamate, serotonin and GABA (Itier and Bertrand, 2001). The nAChR plays a vital role in cholinergic signalling and changes caused by chemical binding or alterations within the structure can have an impact on their function. The channel consists of 5 glycoprotein subunits, all with extracellular N- and C- terminals and 4 transmembrane regions (M1-M4) (Fig. 1.5A). The mammalian vertebrate nAChR is a heteropentamer made up of the subunits: α, β, γ, δ and ε. In muscle type receptors the subunits α1, β1, γ, δ and ε are present. Foetal muscle contains the γ subunit which is replaced by ε in adults (Millar and Gotti, 2009). Neuronal receptors can consist of subunits α2-10 and β2-4. The α-subunits α7, α8 and α9 are able to form homopentamers which are sensitive to α-Bungarotoxin (α-Bgt). The α2-α6 subunits and α10 require a β subunit or another α-type subunit to form a functional channel (Karlin, 2002). The α8 subunit is found only in birds. In insects the subunits are classified into α and non-α (or β) subunits. Each subunit has 4 hydrophobic segments thought to traverse the membrane (M1-M4). The subunits have long N-terminals in the extracellular site with glycosylation sites, a large intracellular domain between M3 and M4 with phosphorylation sites and a short C-terminal (Itier and Bertrand, 2001). The α-subunit is defined as having 2 adjacent cysteine residues in loop C in the N-terminal, essential for ACh binding (Sattelle et al., 2005). Subunits without these cysteines are non α-subunits. The ACh binding site is found over 2 adjoining α and α/β subunits, or α and δ/γ in muscle. ACh binds to cysteine and aromatic residues in a ‘hairpin’ loop thought to consist of β-sheets (Corringer et al., 2000). Binding of ACh causes a conformational change which leads to the opening of the gate via rotation of the M2 regions which line the pore (Miyazawa et al., 1999). The nAChR can exist in 4 states: resting (closed), open, fast onset desensitized and slow onset desensitized.

To date ten genes encoding nAChR subunits have been identified in the Drosophila genome. Seven of these subunits are classified as α, and three as β subunits. In
other insects the orthologs of Dβ2 are α subunits, suggesting a change in function in *Drosophila*. Da5, Da6 and Da7 show the highest homology to vertebrate α7 (42-46%). The remaining *Drosophila* subunits most closely resemble the vertebrate α2 subunit, with Da1 showing highest homology (38-40%) and Dβ3 the lowest (12-22%) (Jones and Sattelle, 2010). Dβ3 is the most distantly related to the other subunits (Fig1.5B). This subunit is a ‘divergent’ subunit, something that is found in most insect species. The divergent subunit shows low homology to all other nAChR subunits and has a small intracellular domain between TM3 and TM4. The divergent subunits may play species specific roles as insect species studied display different sets of these subunits (Jones and Sattelle, 2010).

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**Figure 1.5** Insect nAChR and subunit relationships

**A.** Schematic representation of the nAChR as it is positioned in the membrane. ACh is bound and the channel is in the open confirmation, allowing cations to move into the cell. Adapted from Jones and Sattelle (2010). **B.** Tree showing relationships of *Drosophila* nAChR subunits (α in blue and β in red) and vertebrate nAChR subunits (vert). Adapted from Sattelle *et al* (2005).
The nAChR is implicated in many neuronal diseases. In Alzheimer's disease expression of the subunit α4 decreases by more than 80% (Albuquerque et al., 2009). Additionally, the nAChR plays an important role in addiction, for example, repeated exposure to nicotine leads to the upregulation of high-affinity α4β2 expression. This is thought to be due to reduced receptor function because of desensitization. α7 and α4 are thought to be involved and β3 and/or β4 may convey tolerance to nicotine (Albuquerque et al., 2009). A single inherited trait of schizophrenia has been linked to a locus near the α7 gene and schizophrenic patients have fewer high affinity nAChRs (Weiland et al., 2000). The subunits α4 and α2 have been implicated in autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE). Mutations in the muscle α, β, δ and ε subunits are linked to muscle weakening congenital myasthenia syndromes (Jones and Sattelle, 2010). In Drosophila the Da7 subunit has an excitatory role and is important in the adult escape response (Fayyazuddin et al., 2006; Ping and Tsunoda, 2012). A reduction of Da1 has been linked to decreased amplitude of miniature postsynaptic events (Pym et al., 2006).

The metabotropic ACh receptor, mAChR, is a class of heptahelical G-protein coupled receptors that activate secondary messenger pathways and can be activated by muscarine. The effects on the target cell can be excitatory or inhibitory. They are important in regulating heart rate, smooth muscle contraction, glandular secretion as well as many functions in the CNS (Kruse et al., 2014). The mAChR consists of 7 transmembrane domains with 2 intracellular loops and 3 extracellular loops (Hulme et al., 1990; Kruse et al., 2012). In mammals there are 5 subtypes of receptor M1–5. M1, M3 and M5 are coupled to the Gq/11 family of G-proteins and M2 and M4 are coupled to Gi/0 family. Gq/11 leads to activation of phospholipase C-β and an intracellular IP3/Ca2+ cascade that is mostly excitatory. Activation of M1, M3 and M5 receptors can lead to excitatory effects on the target cell by, for example, inhibition of potassium channels or activation of cation channels. Gi/0 leads to inhibition of adenylate cyclase, causing decreased intracellular cAMP and/or leads to opening of G-protein coupled potassium channels mediated by the βγ subunit of Gi/0. Activation of M2 and M4 can lead to inhibition or hyperpolarisation by activation of potassium channels or inhibition of calcium channels (Brown, 2010; Ren et al., 2015). In mammals mAChR are found in the parasympathetic nervous system and also throughout the CNS where they play a role in learning, memory and reward. They are therefore also relevant in diseases such as Alzheimer's and drug addiction (Kruse et al., 2014; Ren et al., 2015). Drosophila has two types of mAChR: A and B.
Type A is structurally and pharmacologically similar to the mammalian $M_{1-5}$ types and is coupled to the $G_{q/11}$ second messenger pathway. Type B is 1000-fold less sensitive to muscarine than type A and is coupled to $G_{i/o}$ secondary messenger pathway (Collin et al., 2013; Ren et al., 2015). mAChR are most prevalent in the nervous system of insects and expression in adult Drosophila is found in antennal lobes and mushroom bodies (Blake et al., 1993; Hannan and Hall, 1996). In larvae expressing mAChR_A-GAL4 driven expression of GFP mAChR is found throughout the CNS (Silva et al., 2015).

ACh in the synaptic cleft must be removed to prevent continual activation of receptors. AChE is the enzyme responsible for hydrolysis of ACh. ACh is broken down into choline and acetate (Fournier et al., 1987; Silman and Sussman, 2005). AChE has a fast rate of turnover and hydrolysis only takes a few milliseconds, this may be facilitated by the strong electric field of this protein attracting ACh and expelling acetate. AChE can be alternatively spliced into three forms: synaptic (AChE-S), erythrocytic (AChE-E) and readthrough (AChE-R). AChE-S can form tetramers and attach covalently to hydrophobic P-subunits or T-subunits that allow attachment to membranes. T-subunits are able to cluster AChE into bundles of 4, 8 or 12 (Soreq and Seidman, 2001). AChE-R production is induced by stress and does not have any features that allow attachment to other molecules and so is assumed to remain monomeric and soluble (Zimmerman, 2013).

In many insects there are two genes that encode AChE but in Drosophila there is only one, termed $ace$ (Kim et al., 2011). Choline can be recycled back into the presynaptic neuron via sodium dependent high affinity choline transporters at the plasma membrane. This choline can then be used to create another ACh molecule by ChAT (Arvidsson et al., 1997). Choline uptake is a rate limiting step in the SV cycle and is regulated by neuronal activity; for example, choline uptake is upregulated in ChAT deficient mice (Brandon et al., 2004; Bazalakova and Blakely, 2006).

A system in which central cholinergic synapses can be easily studied, such as Drosophila larvae, may provide a tractable model for studying neuronal disease where components of this synapse are disrupted. This may also be a good model to study drugs or insecticides that regulate cholinergic signalling. Insecticide classes have many targets within the nervous system including those at the cholinergic synapse. In the next section classes of insecticides and their necessity are discussed.
1.2 Insecticides

The need for pest control has never been more critical: the world population is predicted to reach 7.7 billion by 2020 and there is limited suitable farmland for growing food. Pathogens, weeds and animal pests reduce potential crop yield. Without insecticides and nematicides, animal pests would cause a loss of 18% of potato crops, 24% of rice crops, 14% of maize and 9% of wheat worldwide (Oerke and Dehne, 2004). Insecticides also have a valuable role in the control of insect disease vectors such as those that carry malaria, dengue and chagas disease (Zaim and Guillet, 2002). Insecticides in some form have been used for thousands of years, but only in the past century have synthetic compounds become the dominant source of insecticides (Casida and Quistand, 1998; Fishel, 2009). Previously plant derived compounds were the best source of insect control, but they have low photostability. One of the first synthetic insecticides was DDT in 1939, closely followed by other chlorinated hydrocarbon compounds hexachlorocyclohexans and lindane in 1942. Unfortunately, these compounds caused environmental problems due to their persistence and bioaccumulation (Casida and Quistand, 1998). Other classes of early insecticides were the organophosphates and the carbamates. These were less persistent than the chlorinated compounds and advanced development of more targeted insecticides. This sections aims to give an overview of current insecticides available and problems faced. I will focus on neurotoxic insecticides because they are relevant to this thesis, but it should be noted that there are many insecticides that target growth regulation and metabolism (IRAC, 2015). A brief summary of current neurotoxic insecticide classes and modes of action are given below and summarised in table 1.1. Insecticide classes are listed as mode of action defined by the IRAC mode of action classification scheme (2015), however some insecticidal compounds have been reported to have more than one mode of action.

Current commercial insecticides target two sites at the cholinergic synapse: AChE and nAChR. The carbamates and organophosphates are inhibitors of the AChE and bind to the ACh binding site of the enzyme to inhibit its function. This leads to accumulation of ACh in the synaptic cleft. Initially this causes prolonged opening of the nAChR and so excitation of the nervous system, however once the nAChR desensitises, paralysis occurs (Koelle, 1994; Pohanka, 2002; Casida and Durkin, 2013). Neonicotinoids, nicotine, sulfoxaflor and butenolides are competitive modulators of the nAChR. They compete with ACh and cause increased opening of
the channel. Eventually this leads to desensitisation of the receptor and paralysis (Fishel, 2005; Matsuda et al., 2005; Casida and Durkin, 2013). Spinosyns are allosteric modulators of nAChR that have specificity for the Da6 subunit. Spinosyns also prolong opening of the channel, again causing increased excitation and eventual paralysis (Salgado, 1998; Perry et al., 2007). The Nereistoxin analogues block the nAChR leading directly to paralysis (Yamamoto and Casida, 1999; Lee et al., 2003).

Other ion channel targets of insecticides are the voltage gated sodium channel (VGSC), GABA-gated chloride channel and glutamate-gated chloride channel. The pyrethroids, pyrethrins, DDT and methoxychlor are modulators of the VGSC. VGSCs are responsible for the initiation of action potentials. These modulatory insecticides prolong the open state of the sodium channel, leading to increased excitation. This is characterised by hyperexcitability and uncoordination. Eventually, depletion of neurotransmitter leads to paralysis, an effect known as ‘knockdown’ (Davies et al., 2007; Casida and Durkin, 2013). Indoxacarb and metaflumizone block the VGSC, preventing conduction of sodium and leading to a state known as pseudoparalysis, where the insect appears paralysed but responds to mechanical stimuli. Eventually as more sodium channels are blocked complete paralysis and death occurs (Lapied et al., 2001; Salgado and Hayashi, 2007). The cyclodiene organochlorines and phenylpyrazoles are antagonists of GABA-gated chloride channels. These channels mediate inhibitory signalling in the insect nervous system. When blocked, increased excitation occurs followed by paralysis due to depletion of neurotransmitters. Fipronil, a phenylpyrazole also inhibits glutamate-gated chloride channels and glycine receptors (Ware, 2004; Zhao et al., 2005; Islam and Lynch, 2012; Casida and Durkin, 2013). Avermectins and mildemycins are allosteric modulators of glutamate-gated chloride channels, these channels are also involved in inhibition in the nervous system. These insecticides increase chloride conductance of the channels, leading to paralysis. Some of these allosteric modulators have also been reported to act on GABA-gated chloride channels (Wolstenholme and Rogers, 2005; Fanigliulo and Sacchetti, 2008).

Other insecticide targets within the nervous system are octopamine and ryanodine receptors. Amitraz is an agonist of the octopamine receptor. Octopamine in insects acts as a neurotransmitter controlling endocrine gland activity, a neurohormone and a neuromodulator. Amitraz causes tremors and convulsions and also influences feeding and egg laying (Casida, 2009; Prullage et al., 2011). The diamides are modulators of the ryanodine receptor. The ryanodine receptor mediates the release
of calcium from intracellular stores (Meissner, 1994). The diamides activate ryanodine receptors, leading to depletion of internal calcium stores. This interferes with muscle contraction and results in paralysis (Cordova et al., 2006; Sattelle and Cordova, 2008).

**Table 1.1 Neurotoxic insecticide classes and modes of action (IRAC, 2015).**

<table>
<thead>
<tr>
<th>Primary target site</th>
<th>Mode of action</th>
<th>Sub-group</th>
<th>Example Active compound</th>
</tr>
</thead>
<tbody>
<tr>
<td>AChE</td>
<td>inhibitors</td>
<td>Carbamates</td>
<td>Aldicarb</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Organophosphates</td>
<td>Malathion</td>
</tr>
<tr>
<td>nAChR</td>
<td>competitive modulators</td>
<td>Neonicotinoids</td>
<td>Imidacloprid</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Nicotine</td>
<td>Nicotine</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sulfoxaflor</td>
<td>Sulfoxaflor</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Butenolides</td>
<td>Flupyradifurone</td>
</tr>
<tr>
<td></td>
<td>allosteric modulators</td>
<td>Spinosyns</td>
<td>Spinosad</td>
</tr>
<tr>
<td></td>
<td>blockers</td>
<td>Nereistoxin analogues</td>
<td>Cartap hydrochloride</td>
</tr>
<tr>
<td>VGSC</td>
<td>modulators</td>
<td>Pyrethroids/pyrethrins</td>
<td>Deltamethrin</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DDT/methoxychlor</td>
<td>DDT</td>
</tr>
<tr>
<td></td>
<td>blockers</td>
<td>Indoxacarb</td>
<td>Indoxacarb</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Metaflumizone</td>
<td>Metaflumizone</td>
</tr>
<tr>
<td>GABA-gated chloride channel</td>
<td>blockers</td>
<td>Cyclodiene organochlorines</td>
<td>Chlordane</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Phenylpyrazoles (Fiproles)</td>
<td>Fipronil</td>
</tr>
<tr>
<td>Glutamate-gated chloride channel</td>
<td>allosteric modulators</td>
<td>Avermectins/Mildemycins</td>
<td>Emamectin</td>
</tr>
<tr>
<td>Octopamine receptor</td>
<td>agonist</td>
<td>Amitraz</td>
<td>Amitraz</td>
</tr>
<tr>
<td>Ryanodine receptor</td>
<td>modulators</td>
<td>Diamides</td>
<td>Flubendiamide</td>
</tr>
</tbody>
</table>

This long list of insecticides and targets appears sufficient to tackle any pest population. However modern insecticides are under more scrutiny than ever before over their impact to the environment and human health. New insecticides must pass
rigorous vertebrate and mammalian toxicity tests, be effective at low concentrations and have a relatively short half-life so as not to persist in the environment (Casida and Quistad, 1998). Further increasing health concerns are studies linking insecticides to Parkinson’s disease (Kamel, 2013; Pezzoli and Cereda, 2013; van der Mark et al., 2014). Designing insecticides that are specific to insect targets would help, for example the AChE has an insect specific cysteine residue that could provide a potential target (Pang et al., 2012). The impact of insecticides on non-target invertebrates, particularly essential pollinators, has also come under scrutiny in recent years (Connolly, 2013; Pisa et al., 2015). The effect on the honeybee, *Apis mellifera*, has gained particular attention. Neonicotinoids have been ‘blamed’ for the collapse of some honeybee colonies, leading to the recent ban in Europe (Farooqui, 2013; Gross, 2013; Fairbrother et al., 2014). The problem of insecticide specificity can only be tackled by making insecticides specific to their target pest. This could be achieved by targeting a protein or area of a protein unique to that pest, for example the divergent subunit of nAChR (Jones and Satelle, 2010). Or designing insecticides that are metabolised to an active compound by enzymes only found in target pest species. Another problem facing insecticides that render them ineffective is the rise of resistance in pest populations. Resistance can be target site or metabolic (Feyereisen, 1995; Ffrench-constant et al., 2004). Target site resistance occurs when a mutation in the target protein prevents or alters binding of the insecticide, so reducing its effectiveness. For example, mutations in the sodium channel have led to DDT and pyrethroid resistance (Williamson et al., 1993; 1996). Metabolic resistance involves the upregulation of genes such as cytochrome P450, aliesterases and other hydrolyses and glutathione S-transferases, that enhance detoxification of the insecticide. For example, amplification of a cytochrome P450 gene in the Aphid *Myzus persicae* underlies neonicotinoid resistance (Scott, 1999; Puinean et al., 2010).

Resistance occurred soon after the introduction of widely used synthetic insecticides. In the 1940s pests resistant to DDT, cyclodiene and organophosphates developed (Sparks and Nauen, 2015). Insecticides put a selection pressure on the pest population. If an individual has a mutation or upregulation of metabolic genes, this provides a fitness advantage over the rest of the population. Therefore, this insect is more likely to breed and pass on its resistance genes. This problem is often exacerbated by farmers increasing the frequency or dose of insecticide applied (Casida and Quistad, 1998; Biezla, 2008). Tackling the problem of resistance requires a range of tactics. These include: 1)
rotation of insecticides with different modes of action/ mechanisms of resistance. 2) biological control such as predators or pathogens, 3) genetic control, for example a newly developed method of expressing RNAi against insect targets in the host plant, and 4) biotechnical control such as pheromones or colour traps (Biezla, 2008; Fu et al., 2014; Koch and Koqel, 2014; Sparks and Nauen, 2015). These methods are aimed to reduce the selection pressure on pests and so prevent or slow down emergence of resistance.

The development of new insecticides with novel target sites is of initial importance. An exciting new target for insecticides that has yet to have a commercial product on the market is the VAChT. The first class of insecticidal compound with this target is the spiroindoline compounds (includes 5Cl- and 5F-CASPP), developed by Syngenta (Sluder et al., 2012). In lepidopteron larvae (order includes moths and butterflies) the spiroindoline compounds caused reduced feeding activity followed by complete paralysis. The binding site for spiroindolines was located in the head membranes of *Lucillia* (blow fly) and found to be present across insect orders. Displacement experiments using other insecticides showed these compounds to have a novel target (Sluder et al., 2012). The target site was narrowed down to the cholinergic synapse by observations that the spiroindolines supressed the effects of aldicarb (an AChE inhibitor) in *C.elegans*. A mutation screen using *C.elegans* grown on spiroindoline containing media identified 10 strains that were resistant to the insecticide, 7 of these had dominant mutations that mapped to chromosome 3 and were point mutations in unc-17. UNC-17 encodes the *C.elegans* VAChT. Confirmation of VAChT as the target site was verified in *Drosophila* by the observations that 1, upregulation of VAChT using fly transgenics results in ~3 times greater resistance to spiroindoline compounds. 2, Flies overexpressing a mutant Y49N form of the VAChT were >10 times more resistant than control flies. Finally, VAChT transfection into PC12 cells facilitates spiroindoline compound binding and a dose dependent inhibition of ACh uptake (Sluder et al., 2012). However, no in vivo evidence of inhibition of ACh uptake has been obtained. The discovery of the spiroindoline compounds highlights the value of *Drosophila* in understanding mode of action of new insecticides. In the following section I will discuss the *Drosophila* nervous system and aspects that will be utilised in this thesis.
1.3 *Drosophila* nervous system

The life cycle of *Drosophila* is completed over 10 days at 25°C. Development of the embryo takes 24 hrs, and first and second instar stages last approximately one day each. The third larval stage (third-instar) continues for around 3 days before pupation occurs. The pupal stage lasts 4 days before the adult fly emerges and adult flies reach sexual maturity after 2-4 days (Chyb and Gompel, 2013). This project utilises third-instar larvae and adult flies in electrophysiological assays.

In *Drosophila* larvae the brain consists of two brain lobes and the ventral nerve cord (VNC). The VNC contains three thoracic segments (T1-3) and ten abdominal segments (A1-10) (Fig. 1.6A,B). ACh and glutamate are the principal excitatory neurotransmitters in the CNS and NMJ respectively. Other neurotransmitters and neuromodulators include: serotonin, dopamine, histamine, octopamine, GABA, taurine and aspartate (Monastirioti, 1999; Prokop and Meinertzhagen, 2006). Neurons of the *Drosophila* CNS first show a response to ACh at 13 hrs After Egg Laying (AEL), but a developmental role other than as a neurotransmitter is suggested at this stage. Currents which are characteristic of endplate synaptic currents (EPSCs) are first seen at 16 hrs AEL and action potentials at 17 hrs (Baines and Bate, 1998). As well as being strongly excited by ACh, motoneurons also respond to GABA and glutamate, though more weakly and produce inhibitory inward chloride currents (Rohrbough and Broadie, 2002). GABA is thought to be the main inhibitory neurotransmitter in the insect CNS.

The system that controls locomotion in larvae is amenable to study and output from this system can be measured at the motoneuron and muscular level. Locomotion is driven by a central pattern generating circuit (CPG) that drives the typical pattern of muscle movement seen in peristaltic crawling of larvae (Grillner, 2006; Crisp *et al.*, 2008). The term central pattern generator can be used to describe a group of neurons that control a stereotypical pattern of behaviour, often groups of interneurons that control the activity of motoneurons. Controlled behaviour can include locomotion, respiration and swallowing (Arshavsky *et al.*, 1997; McCrimmon *et al.*, 2000). The assembly of neurons which set up the CPG, and so drive locomotion in *Drosophila* larvae, is pre-determined and does not require sensory input (Suster and Bate, 2002). The CPG interneurons innervate motoneurons in the VNC: there are 36 motoneurons per half segment in the larval VNC, derived from 15 of the 30 neuroblasts (NB) (Baines and Pym, 2006; Landgraf and Thor, 2006). The CPG interneurons are believed to be cholinergic and motoneuron dendrites within
the VNC neuropil overlap with cholinergic axonal fibers, and a downstream excitatory response is seen in the motoneurons (Rohrbough et al., 2000; Rohrbough and Broadie, 2002; Baines, 2003). Motoneuron axons project through 3 principal nerves into the muscle field: the intersegmental (ISN) and segmental nerve (SN) and the transverse nerve (TN) which contains 2 motor axons and projects along the segment border (Landgraf and Thor, 2006). The motoneurons are glutamatergic and innervate the body wall muscles (Fig. 1.6C). Drosophila larvae have segmentally repeated sets of body wall muscles (30 per abdominal half segment) that power movement of the denticle belt over the substrate by waves of contractions. Muscle movement in Drosophila can be seen in the embryo before hatching. Initially muscle twitches are myogenic as they are unaffected by blocking neural activity. By 18.25 hrs AEL the sequence of muscle contractions resembles complete waves of larval crawling (Crisp et al., 2008). The sensory input to the locomotion patterns appears to be fully integrated only during later stages of embryogenesis.

Figure 1.6  Drosophila CNS in third-instar larvae and locomotive neural pathway

A. The larval CNS as it is positioned within the larvae (purple), anterior is on the left and mouth hooks are shown. Adapted from Hertenstein (1993). B. Schematic of larval CNS showing brain lobes, VNC and dorsal motoneurons. Adapted from Glanzman (2010). C. Neural pathway from the CPG neurons (blue) that form cholinergic synapses with motoneurons (green). The motoneurons form glutamatergic synapses onto the body wall muscles (pink).
Motoneuron output can be measured by recording EJPs from the body wall muscles and input to the motoneurons can be measured by whole-cell patch-clamp of these neurons. Individual motoneurons can be identified by their cell body position and distinctive dendritic and axonal projections. Specific motoneurons can also be visualised by neuron specific GAL4 lines. Two well characterised motoneurons utilised in this thesis are the dorsal motoneurons aCC, generated from neuroblast NB1-1 and RP2, from NB4-2 and innervating muscles 1 and 2 respectively (Fig. 1.7, Landgraf et al., 1997).

**Figure 1.7** Schematic representation of aCC and RP2 motoneurons and their axonal projections to the muscle

The embryonic motoneurons are shown with their central and peripheral projections and muscle targets. Motoneuron name is top left of motoneuron and bottom right is the nerve through which axons project: anterior root of ISN (ISNa), posterior root of ISN (ISNp). Muscle names are above muscle. AC = anterior commissure, PC = posterior commissure. Adapted from Landgraf et al (1997).

Since their identification in *Drosophila* by homology to identified grasshopper neurons (Thomas et al., 1984), knowledge about aCC and RP2 is steadily increasing. Patch-clamp recordings in the embryo found that aCC and RP2 have an inward sodium current with transient and persistent components (Na\(_T\) and Na\(_P\)), two
outward potassium currents ($K_{\text{fast}}$ and $K_{\text{slow}}$), at least two inward voltage dependent calcium currents ($I_{\text{Ca}}$) and ACh gated inward currents ($I_{\text{ACH}}$) (Baines and Bate, 1998). The neuronal identity of these neurons is determined by transcription factors even-skipped (eve), grain and the zinc finger homeobox gene zfh1. They do not express Islet, Lim3 or Hb9 which are transcription factors found in the ventral motoneurons and influence ion channel gene expression (Landgraf and Thor, 2006, Wolfram et al., 2012, 2014). Eve expression is important in determining axonal projection to the muscle and also influences $K_{\text{fast}}$ and $I_{\text{ACH}}$ (Garces and Thor, 2006; Pym et al., 2006). There is no difference in synaptic input between these two motoneurons and synaptic connections are made in the absence of synaptic transmission (Baines et al., 1999; Baines et al., 2001; Pym et al., 2006). Synaptic input to these neurons regulates excitability by altering VGSC expression. This is through the translational repressor activity of Pumilio acting on the VGSC para mRNA (Mee et al., 2004). Alternative splicing of the VGSC gives rise to 27 splice variants some of which influence activation kinetics and Na$_r$ (Lin et al., 2009). In this project I used patch-clamp recordings from aCC and RP2 to investigate action potential evoked and spontaneous ACh release when VAChT is blocked by the spiroidoline insecticide 5Cl-CASPP and when VAChT is overexpressed. Action potential evoked fusion of SVs leads to large inward currents at the postsynaptic motoneuron termed spontaneous rhythmic currents (SRCs). These often result in action potentials in the motoneuron. Figure 1.6A provides an example of these currents recorded under voltage-clamp. Action potential independent fusion of single SVs leads to characteristic small inward currents, termed minis (Fig. 1.6B). Analysis of minis provide information concerning how VAChT expression relates to quantal release of ACh. SRCs by initiating action potentials directly influence output of the motoneuron to the muscle and so a change here may reflect an effect on behaviour. The ability to identify the same neurons in different animals and ease of access to central cholinergic synapses offers a major advantage of this system.
The CNS of the adult *Drosophila* can be divided broadly into the central brain, the optic lobes and the VNC (Fig. 1.9A). The central brain contains the olfactory centres for learning and memory, termed mushroom bodies (Sousa-Nunes et al., 2010). The central brain and optic lobes are located in the head and the VNC is in the thorax. There is a neuronal network amenable to study that mediates the fly’s escape response. When the fly perceives a possible threat in the form of an overhead shadow, the escape response is activated. This response is mediated by a well characterised network of neurons: the giant fiber system (GFS). During voluntary flight the wings are first elevated from the resting position before the fly extends its legs to jump into the air. During an escape response the pre-flight phase of wing extension is lost, saving ~3 ms. The fly jumps into the air with wings still folded in the resting position and is airborne before they are fully unfolded (Allen et al., 2006).

This neuronal network includes the giant fiber neuron (GF) whose axon is 6-8 µm in diameter in *Drosophila*, huge in the context of the fly’s size. There are 2 bilaterally symmetrical giant fibers which contribute to the multicomponent neural circuit. (Fig. 1.9B) (Allen et al., 2006). The two large GFs relay information from the brain to the thoracic ganglia. Here electrochemical synapses are made with the tergotrochanteral motor neuron (TTMn) which drives extension of the leg muscle (TTM). Electrochemical synapses are also made with the periphery synapsing interneuron (PSI) which in turn has chemical synapses with the dorsal longitudinal muscle motoneuron (DLMn) that innervates the DLMs (Fig. 1.8C), one of the major indirect flight muscles along with the dorsoventral muscles (DVMs) (Allen and

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**Figure 1.8** Synaptic inputs to aCC/RP2 motoneurons recorded under voltage-clamp

A. Action potential dependent spontaneous rhythmic currents (SRCs) in third-instar larvae. B. Action potential independent miniature synaptic currents (minis) in third-instar larvae. Traces were obtained during the course of this project.
Murphey, 2007). The di-synaptic activation of the DLMns (via the PSI, Fig. 1.9C) causes a small delay of ~0.5 ms in the timing of contraction of the DLM muscles relative to the TTM. This delay allows for the TTM to first extend the femur and propel the fly off the ground before the flight muscles are activated (Augustin et al., 2011).

**Figure 1.9** *Drosophila* adult CNS and GFS

A. Schematic of the adult CNS showing the central brain (CB), olfactory lobes (OL) and ventral nerve cord (VNC). Within the ventral nerve cord are the thoracic segments (Th1-3) and the abdominal segment (Ab). Adapted from Sousa-Nunes et al (2010). B. The GFs and GCIs are visualised by staining for β-gal in the A307 GAL4 background. Scale bar is 100 µm. Adapted from Phelan et al (1996). C. Schematic showing the GFS network. The GF neurons connected by the GCI are found in the head with axons into the thorax. The GF synapses with the PSI and TTMns. The PSI synapses with the DLMns. The TTMns and DLMns innervate the TTM and DLM muscles respectively. Adapted from Phelan et al (2008).
It was deduced that there are electrochemical synapses in the GFS because in shak-B<sup>2</sup> mutants where gap junctions do not function a less reliable pathway still exists to the TTM. If both gap junctions and cholinergic signalling are blocked in shak-B<sup>2</sup>;cha<sub>ts2</sub> temperature sensitive double mutants the GF-TTMn synapse is blocked at the restrictive temperature (Allen and Murphey, 2007). Therefore, both electrical and cholinergic chemical synapses are present in the GFS. The neuromuscular junctions of the GFS are glutamatergic (Koenig and Ikeda, 2005). Other synapses are present between the 2 GFs: giant commissural interneurons (GCI) connect them in the brain, GF medial collaterals connect them in the thorax and the PSIs are coupled. Fayyazuddin et al (2006) found that Da7 subunits of the nicotinic acetylcholine receptor were required for transmission from PSI to the DLMNs. The neural pathways that activate the muscles can differ depending on the stimulus. Visually evoked responses use the GFS, but noxious olfactory stimuli appear not to use the GFS but another pathway which converges with the GFS at the jump and flight muscle motorneurons. This flight has the same characteristics as voluntary flight (Allen et al., 2006).

The GFS is a model central neural pathway as many of the neurons can be identified by their size, position and morphology and the development of individual neurons can also be mapped (Allen et al, 2006). The GFS can be activated by applying sensory (air-puff or lights-off) stimulus, or by direct application of an electrical stimulus to the brain. These lead to action potentials which reach the TTMNs and DLMNs via the GFs, PSIs and TTMNs/DLMNs (Augustin et al., 2011). Electrophysiological recordings can be carried out by patch-clamp of motoneuron 5 that innervates DLM muscles a and b in situ (Fayyazuddin et al., 2006; Ryglewski and Duch, 2009; Herrera-Valdez et al., 2013). However a simpler technique of recording EJPs from TTM and DLM simultaneously offers a method to monitor the whole system. EJPs can be measured from the TTM and DLM muscles by inserting recording electrodes through the cuticle (Allen and Godenschwege, 2010). Output can be analysed for latency, that is the time delay between stimulation and muscle depolarization and ability to produce EJPs in response to repeated high frequency stimuli. This allows the study of the functional status of the GFS’ components, including central synapses (GF-TTMn, GF-PSI, PSI-DLMn) and chemical neuromuscular junction synapse (TTMn-TTM, DLMn-DLM). This includes cholinergic, glutamatergic and electrochemical synapses. This thesis will assess the GFS as a model to define mode of action and determine if output can be characterised to different insecticide mode of actions.
1.4 Outline of thesis

This project aims to use *Drosophila* larvae to confirm the mode of action of the spiroindoline insecticides and also as a tool to study a central cholinergic synapse. In this thesis an adult model to study insecticide mode of action will be assessed for characteristic outputs under insecticide treatment that indicate mode of action.

The effect of the novel spiroindoline insecticide 5Cl-CASPP, on ACh release at central cholinergic synapses in *Drosophila* larvae is described in chapter 3. Mortality assays were used to assess sensitivity of *Drosophila* to spiroindoline compounds. Larval tracking demonstrated the effect on locomotion and patch-clamp recordings from aCC and RP2 was used to determine the effect on spontaneous and evoked ACh release. Transgenic overexpression of wild-type and resistant VACht were used to confirm target protein. Finally this chapter also includes characterisation of the subunits expressed in the nAChR in aCC motoneurons.

Chapter 4 describes the effect of overexpression of two variants of VACht on spontaneous release of ACh in *Drosophila* larvae and how this is related to synapse morphology and SV number.

Chapter 5 explores the GFS in the adult fly as a model to determine insecticide mode of action. Insecticides with known modes of action were used to characterise output of this system. Novel compounds with unknown modes of action were also tested and compared.

The overall conclusions of using *Drosophila* as a model to study insecticide mode of action are discussed in Chapter 6. Overall findings and implications of this project are also described. Finally, outlook and suggested future experimental work are discussed.
Chapter 2

Materials and Methods

2.1 Fly maintenance

Fly stocks were maintained on standard fly food in vials or bottles at 25°C on a 12 hour light-dark cycle. Fly food was prepared in the Faculty of Life Sciences and contained: 390 g glucose, 360 g maize, 250 g yeast and 50 g agar in 5 litres of water. After heating to 98°C for 10 minutes and allowed to cool, 135 ml of nipagen and 15 ml of propionic acid were added. Larvae which expressed channelrhodopsin2<sup>H134R</sup> (ChR2) were raised in food vials to which 100 µl 0.1M all-trans-retinal (in ethanol, Sigma Aldrich, UK) was added; this is an essential co-factor for light-induced activation of the channel.

2.2 Fly stocks

A list of fly stocks is provided below. To express transgenes in specific cells, homozygous GAL4 driver lines were crossed to UAS reporter lines to produce heterozygous progeny. Where the UAS line was over a balancer chromosome, larvae from the cross were selected against presence of the balancer (using GFP). In the text, these progeny will be referred to as driver>reporter, for example cha<sup>B19</sup>-GAL4 crossed with UAS_VACChT<sup>(Syn)</sup> is referred to as cha>VACChT<sup>(Syn)</sup>.
Table 2.1  Fly stocks used in this study. Abbreviated names used in text are in brackets.

<table>
<thead>
<tr>
<th>Fly stock</th>
<th>Identity</th>
<th>Application</th>
<th>Obtained from</th>
<th>ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>CantonS (CS)</td>
<td>Wild-type</td>
<td>multiple</td>
<td>Bloomington Stock centre</td>
<td>1</td>
</tr>
<tr>
<td>OregonR (OrR)</td>
<td>Wild-type</td>
<td>behaviour, ephys</td>
<td>Prof. Baines (UoM)</td>
<td></td>
</tr>
<tr>
<td>+;+;RRa-GAL4;CD8GFP (RRa-GFP)</td>
<td>GFP</td>
<td>(green fluorescent protein) cell membrane marker in subset of motoneurons</td>
<td>Dr. Lin (UoM)</td>
<td></td>
</tr>
<tr>
<td>+;cha^{Tr};GAL4;+ (cha)</td>
<td>Driver of targeted protein expression in cholinergic neurons</td>
<td>multiple</td>
<td>Prof. Baines (UoM)</td>
<td></td>
</tr>
<tr>
<td>+;+;RRa-GAL4 (RRa)</td>
<td>Driver of targeted protein expression in subset of motoneurons</td>
<td>ephys</td>
<td>Prof. Baines (UoM)</td>
<td></td>
</tr>
<tr>
<td>+;+;UAS_VAChT^T^{RNAi}</td>
<td>Wild-type VACHT</td>
<td>multiple</td>
<td>Dr. Willis (Syngenta)</td>
<td></td>
</tr>
<tr>
<td>+;+;UAS_VAChT^T^{RNAi}</td>
<td>Wild-type VACHT</td>
<td>ephys</td>
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<td></td>
</tr>
<tr>
<td>+;+;UAS_VAChT^Y49N</td>
<td>VACHT resistant to CASPP</td>
<td>behaviour, ephys</td>
<td>Dr. Willis (Syngenta)</td>
<td></td>
</tr>
<tr>
<td>+;+;UAS_VAChT^G342R</td>
<td>VACHT with amino acid change disrupting synaptobrevin interaction</td>
<td>ephys</td>
<td></td>
<td></td>
</tr>
<tr>
<td>y^{V}:P[TRip.JF02764]attP2;+ (VACHT^{RNAi})</td>
<td>RNAi against VACHT</td>
<td>ephys</td>
<td>Bloomington Stock centre</td>
<td>27684</td>
</tr>
<tr>
<td>+;+;VACHT^T/TM6C Sb Tb</td>
<td>VACHT null</td>
<td>ephys</td>
<td>Dr. Willis (Syngenta)</td>
<td></td>
</tr>
<tr>
<td>+;+;UAS_nAChRa7.GFP/TM3 ser act::GFP (nAChR_Da7)</td>
<td>Da7 subunit of nAChR</td>
<td>ephys</td>
<td>Modified from Bloomington Stock centre</td>
<td>39692</td>
</tr>
<tr>
<td>nAChRa6^Tr/Snb</td>
<td>Null for nAChR Da6</td>
<td>ephys</td>
<td>Dr. Willis (Syngenta)</td>
<td></td>
</tr>
<tr>
<td>nAChRa7^Tr/^{DN}</td>
<td>Null for nAChR Da7</td>
<td>ephys</td>
<td>Bloomington Stock centre</td>
<td>24879</td>
</tr>
<tr>
<td>Shi^{Tr}</td>
<td>Temperature sensitive dynamin mutant</td>
<td>ephys</td>
<td>Dr. Matsubayashi (UoM)</td>
<td></td>
</tr>
<tr>
<td>+;UAS_ChR2^{Tr/Ts4}</td>
<td>Channel Rhodopsin</td>
<td>ephys</td>
<td>Prof. Baines (UoM)</td>
<td></td>
</tr>
</tbody>
</table>

ephy = electrophysiology, EM = electron microscopy, UoM = University of Manchester
2.3 Mortality assays

*Larval mortality assays* were carried out at 25°C in standard fly food vials. 100 µl of a 5% w/v yeast extract (Merck, Germany) solution was first added to each vial to prevent drying out. 50 µl of compound or vehicle (acetone) was added to the surface of the food and the surface disturbed with a sharpened pin to aid absorbance. Twenty first-instar larvae were added to each vial. Assays were carried out in triplicate. Lethality was assessed seven days later as number of larvae that did not reach pupation. Compounds used in larval mortality assays were: spiroindoline variants 5CL-CASPP and 5F-CASPP (SYN351 and SYN876 as described in Sluder et al., 2012. Manufactured by Syngenta, Jealott's Hill, UK) which were dissolved in acetone at concentrations 0 to 10 µg/ml and L(−)-vesamicol (supplied by Syngenta) dissolved in ethanol at concentrations 0 to 1000 µg/ml (Fig. 2.1). Stock concentration of compounds were stored at -20°C.

*Acute compound treatment* for tracking and electrophysiology experiments was carried out on 50 mm grape agar plates (Genesee Scientific, USA). Compound or vehicle was included in 1 ml of 5% yeast extract solution. The volume of solution added to the plates was higher than the volume added to vials, therefore the concentration of compound was decreased to ensure total quantity of compound was the same. For example, in vial assays 50 µl of 10 µg/ml 5Cl-CASPP was added which represents 0.5 µg of 5Cl-CASPP. In plate treatment, 1 ml of 0.5 µg/ml 5Cl-CASPP solution was added, also equalling 0.5 µg 5Cl-CASPP. For 5Cl-CASPP treatments 0.5, 0.45 and 0.25 µg were used and in L(−)-vesamicol treatment 50 µg was used. Plates were allowed to dry on a warm plate (~30°C) for 2 hrs. Approximately 30 early third-instar larvae were added to each plate 24 hrs prior to experimental use.

*GFS adult drug applications* were carried out in 24 well plates (ThermoFisher scientific, UK). Wells were filled with 1.5 ml of 1% agar in water (OXOID, ThermoFisher Scientific, UK), 5% sucrose solution and allowed to set. Sucrose was included to promote feeding and contact. Compounds were solubilised in 10% acetone:ethanol (1:1) and 90% hydrofluoroether (HFE). 20 µl of compound or vehicle (acetone:ethanol:HFE, 0.5:0.5:9) was added to each well. HFE was used as it allows the sample to spread across the surface of the agar. Compounds used in feeding/contact assays are listed in Table 2.2 and Fig. 2.1 below. Plates were allowed to dry completely (minimum of 30 min) and 10 flies (1-5 days old) were added to each well. Plates were sealed with a gas permeable membrane (Breath-
easy, Diversified Biotech, USA) and holes punctured with a pin to further aid gas exchange. Mortality was assessed after 24 hrs. Compound doses were initially tested at a broad range (0–1000 µg/ml) and adjusted to give a range causing 0-100% mortality, three doses from this range were then used in recordings. Assays were carried out in duplicates and surviving flies were used in GFS recordings. Chemicals were from Fisher Scientific (UK) unless otherwise stated.

Table 2.2  Compounds used in contact/feeding assays and GFS recordings

<table>
<thead>
<tr>
<th>Compound</th>
<th>Insecticide class</th>
<th>Additional Information</th>
<th>Source</th>
<th>Doses used (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indoxacarb</td>
<td>Voltage dependent sodium channel blocker</td>
<td>Metabolised to a sodium channel blocker</td>
<td>Syngenta</td>
<td>1000</td>
</tr>
<tr>
<td>Decarbomethoxylated JW062 (DCJW)</td>
<td>Voltage dependent sodium channel blocker</td>
<td>Active metabolite of indoxacarb</td>
<td>Syngenta</td>
<td>0.5, 2, 4</td>
</tr>
<tr>
<td>Spinosad (Spinosyn)</td>
<td>Nicotinic acetylcholine receptor (nAChR) allosteric modulators</td>
<td>nAChR Dα6 allosteric modulator</td>
<td>Sigma-Aldrich, UK</td>
<td>8, 16, 32</td>
</tr>
<tr>
<td>Fipronil (phenylpyrazole)</td>
<td>GABA-gated chloride channel blocker</td>
<td></td>
<td>Syngenta</td>
<td>2, 8, 16</td>
</tr>
<tr>
<td>Deltamethrin (pyrethroid)</td>
<td>Sodium channel modulator</td>
<td></td>
<td>Syngenta</td>
<td>0.01, 0.05, 0.5</td>
</tr>
<tr>
<td>CASPP (5Cl-CASPP)</td>
<td></td>
<td>VACHT blocker</td>
<td>Syngenta</td>
<td>1, 5, 10</td>
</tr>
<tr>
<td>Philanthotoxoin-7,4</td>
<td></td>
<td>Glutamate receptor inhibitor</td>
<td>Abcam</td>
<td>3000, 4000</td>
</tr>
<tr>
<td>Compound A</td>
<td></td>
<td>Predicted inhibitors of VACHT (private communication, Syngenta)</td>
<td>Syngenta</td>
<td>50, 100, 300</td>
</tr>
<tr>
<td>Compound B</td>
<td></td>
<td>Suspected allosteric modulator of nAChR Dα6 (private communication, Syngenta)</td>
<td>Syngenta</td>
<td>20, 40, 100</td>
</tr>
<tr>
<td>Compound C</td>
<td></td>
<td>Evidence of activity at housefly NMJ (private communication, Syngenta)</td>
<td>Syngenta</td>
<td>5, 10, 50</td>
</tr>
<tr>
<td>Compound D</td>
<td></td>
<td>Predicted inhibitor of voltage gated sodium channel and low affinity VACHT blocker, (private communication, Syngenta)</td>
<td>Syngenta</td>
<td>4000</td>
</tr>
<tr>
<td>Compound E</td>
<td></td>
<td>Evidence of activity at house fly NMJ (private communication, Syngenta)</td>
<td>Syngenta</td>
<td>100, 500, 1000</td>
</tr>
</tbody>
</table>
**Figure 2.1** Structures of compounds used in this study

Compound structures of spiroindolines and vesamicol used in larval mortality assays and electrophysiology. Structures of commercial insecticides used in GFS study are also shown.

### 1.4 Tracking assays

Third-instar wall crawling larvae were rinsed in distilled water for 30 seconds, gently dried with tissue paper and placed in the centre of a 14 cm petri dish (UKGE, UK) filled with 1% agarose (SeaKem LE Agarose, USA). Larvae were allowed to
acclimatise for 2 minutes before being tracked for a further 2 minutes at room
temperature (20-22°C). Plates were placed on a light-box (model LP812, Jessops,
UK) to enhance contrast of the larvae. A uEye camera (model UI-2230SE-C-HQ,
IDS, Germany) was positioned directly overhead and recordings were made at 5
frames/sec. Distance travelled during 2 min was measured using the tracking
function in Image-Pro plus software (Version 6.3, Media Cybernetics, USA) which
calculated movement using larval centroid body measurements. The raw data was
transferred to Microsoft excel for analysis. Total distance travelled was measured
and peak speed over a distance without stopping or turning of the larvae was
calculated.

2.5 FACS and PCR

Fluorescence activated cell sorting (FACS) was carried out by collecting late third-
instar larval CNSs (~400) from the RRa-GFP genotype; only aCC is labelled by late
stage third-instar. Cells were dissociated using 1 ml of 100 mg/ml
Collagenase/Dispase II (Roche, Germany). The CNSs were triturated in Schneiders
Drosophila medium (Gibco, UK) and strained through a 35 µM nylon mesh filter (BD
Biosciences, USA). GFP cells were sorted using a BD FACSaria™ cell sorter (BD
Biosciences). RNA was extracted using an RNeasy Microkit (Qiagen) in 12 µl
RNase-free water. cDNA was synthesised in a total volume of 22 µl. Primers
Oligo(dt) (0.5 µg) and random hexamers (0.2 µg) were mixed with the RNA before
heating to 65°C for 5 minutes. The RNA was then transferred to ice for 1-2 minutes.
5X reaction buffer (250 mM Tris-HCl, 250 mM KCl, 20 mM MgCl₂, 50 mM DTT) and
dNTP (10 mM) were added to the RNA, together with an RNase inhibitor (20 u/µl
RiboLock™) and reverse transcriptase enzyme (200 u/µl RevertAid™). The reaction
mixture was then incubated at 25°C for 10 minutes followed by 42°C for 1 hr and
finally 70°C for 5 minutes to stop the reaction. PCR for the ten known nAChR
subunits was carried out using primer-pairs specific for each subunit (Table 2.3).
The house keeping gene rp49 was amplified to normalise band intensity in
electrophoresis gels. Control genes (Lim3, Islet, Eve, VGLUT and Ddc) were also
amplified. Reactions were carried out in a total of 20 µl. Primers and template cDNA
were combined with 10 x NH₄-reaction buffer, MgCl₂ (50 mM), dNTP mix (100 mM),
Taq polymerase (BIOTAQ™ DNA Polymerase, BIOLINE) and made up to 20 µl
using ddH₂O. Temperature cycles for the reaction were as follows: denature at
94°C, annealing at 56°C and extension at 72°C. The reaction was carried out for 30 cycles at these temperatures. Electrophoresis was carried out using a 2% agarose (SeaKem, UK) gel and 5 µl of PCR product run at 100 mV. The intensity of the bands was analysed using ImageJ software (1.47v; USA).

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Da1</td>
<td>GAAATGGAGAAGACCACAATA CGA</td>
<td>GAAGATCCACAGAATACCATACGA</td>
</tr>
<tr>
<td>Da2</td>
<td>CGTCATGTTATACAGCACC</td>
<td>CCAAGGATGCCATCATAAAGAG</td>
</tr>
<tr>
<td>Da3</td>
<td>GAACCTGAAGGATCAATACCA</td>
<td>ATCAGGTTGTGATAGTGCAC</td>
</tr>
<tr>
<td>Da4</td>
<td>CCACCAGCAATATAAGAATACCAATC</td>
<td>GTTAGTCATATCTGTGCCTC</td>
</tr>
<tr>
<td>Da5</td>
<td>CAGCAGCAGCAGCAGCAATATT</td>
<td>GGTAGTGGCCATCATAATCCT</td>
</tr>
<tr>
<td>Da6</td>
<td>AGTGGAAACGACTACAATCTCGA</td>
<td>CAATGTTGTGATACGTG</td>
</tr>
<tr>
<td>Da7</td>
<td>CCCTGGTGAGGATAGAACGA</td>
<td>CGTAGGTCCACGAACAAAT</td>
</tr>
<tr>
<td>Dβ1</td>
<td>CGACCCGGTACAGAATATGAC</td>
<td>GTACCAAACCAAACGTAACC</td>
</tr>
<tr>
<td>Dβ2</td>
<td>GTACCAAACCAAACGTAACC</td>
<td>CAACTTGCACTAGGTACTG</td>
</tr>
<tr>
<td>Dβ3</td>
<td>CAACTTGCACTAGGTACTG</td>
<td>CCGTAGTATGTCCGTAGTGAACAA</td>
</tr>
<tr>
<td>RP49</td>
<td>CCAGTCGGATCGATGATATGCTA</td>
<td>ACGTTGTGACACCCAAAG</td>
</tr>
<tr>
<td>Lim3</td>
<td>CACCAGCAATATCTGACAAAGG</td>
<td>ATGCCTTACTGGCATGCA</td>
</tr>
<tr>
<td>Islet</td>
<td>GCCTGGAAGAGCTTAAAGCGCATTT</td>
<td>GATTTGTGCTACGCTGACGTTAG</td>
</tr>
<tr>
<td>Eve</td>
<td>ATACCAAACACAAGGAGAC</td>
<td>CGGGACGATGATGTCTGT</td>
</tr>
<tr>
<td>VGLUT</td>
<td>ATGAGGTGCAATATGTCGCGG</td>
<td>CGCCAGATACGAGCA</td>
</tr>
<tr>
<td>Ddc</td>
<td>ATGGGGCGCGCGGAAGGCAATTC</td>
<td>ACTCAGCAGTCGCTCAGG</td>
</tr>
<tr>
<td>VACHT</td>
<td>CTCACTGGGTAATATGTTGA</td>
<td>ACGGTATGATCTTTCC</td>
</tr>
<tr>
<td>ChAT</td>
<td>ATCACCGAGGGGATGAC</td>
<td>TATAGTGGCCATCGT</td>
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</table>

Primers used to create VACHTG342R sequence

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primer</td>
<td>GATCGGCGGCAGCCCTGCTCATTCA</td>
<td>GCATTTAAATATAATATGCTGCTA</td>
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<tr>
<td>Primer</td>
<td>GCCCTCGGAGCGCTGCTCCTCCTG</td>
<td>CAGGGAAGCCGCTCCAGGC</td>
</tr>
</tbody>
</table>

Primers for sequencing of plasmid

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>T3</td>
<td>ATTAACCCCTCATAAAGGGG</td>
<td></td>
</tr>
<tr>
<td>T7</td>
<td>TAATACGACTACAGTATAGGG</td>
<td></td>
</tr>
</tbody>
</table>

qRT-PCR was carried out using 3 or 5 independent samples of 20 CNSs, collected for control and test groups. After RNA extraction (QIAGEN RNeasy Micro Kit), cDNA was synthesized using the Reverse Aid H minus First strand cDNA synthesis kit (Fermentas, UK). qRT-PCR was performed using a Roche LightCycler480 II (Roche) using the LightCycler SYBRG Master reaction mix (Roche). The thermal
profile used was 5 min at 95°C followed by 45 cycles of 10 s at 95°C, followed by 10 s at 60°C, and finally 10 s at 72°C. Each sample was run in triplicate and an average Ct value calculated per sample. Ct is cycle threshold and indicates the number of thermal cycles before fluorescence is detected. Reference gene was rp49. Results were analysed by the Δ(ΔCt) method. The Δ(ΔCt) method is performed by calculating the difference in Ct values between target gene (VACHT or ChAT) and reference gene (rp49) in each sample to give ΔCt values for test (cha>VACHT+) and control (cha+/+) samples. The mean ΔCt for the control group is then subtracted from each test ΔCt and control ΔCt, to give Δ(ΔCt) values. This is represented in the equations below where cha>VACHT+ and cha/+ represent test and control samples, target and ref represent RNA amplified.

\[
\text{Cha}>\text{VACHT}^+ \Delta(\Delta\text{Ct}) = (\text{Ct (cha}>\text{VACHT}^+, \text{target}) - \text{Ct (cha}>\text{VACHT}^+, \text{ref})) - \text{mean (Ct(cha+)/+, target) - Ct (cha+)/+, ref})
\]

\[
\text{Cha}/+ \Delta(\Delta\text{Ct}) = (\text{Ct (cha+)/+, target}) - \text{Ct (cha+)/+, ref})) - \text{mean (Ct (cha+)/+, target) - Ct (cha+)/+, ref})
\]

To calculate a relative fold change in expression, each Δ(ΔCt) value is transformed using \(2^{-\Delta(\Delta\text{Ct})}\). This method allows change in gene expression to be presented as relative to a control expression of 1. The data can then also be expressed as change in gene expression when control is 0 by transforming the data using log2.

### 2.6 Creation of VACHT\(^{(M)}\) and VACHT\(^{G342R}\) UAS lines

To create UAS lines for the overexpression of VACHT\(^+\) and VACHT\(^{G342R}\), the wild-type (CS) VACHT (VACHT+) sequence was used as a template. RNA was extracted and cDNA created as previously described and together with the primers were combined with 10 mM dNTP, 10 X Phusion HF reaction buffer (Biolabs, UK) and Phusion Taq polymerase (Biolabs, UK). The thermal profile used was 5 min at 98°C followed by 35 cycles of 30 s at 98°C, 30 s at 55°C and 2 min at 72°C. Two different VACHT sequences were required: 1) The wild-type sequence which was amplified using Primers A and D, and 2) VACHT\(^{G342R}\) which required a G to C nucleotide change to be introduced at position 1025 that would translate to the G342R (GGC to CGC) substitution. This was achieved by amplifying the first 1kb of the sequence.
using primers A and B, and the remaining 0.7kb using primers C and D (Table 2.3), primer C introduced the G to C nucleotide change (Fig. 2.1). The PCR products from both of these reactions were precipitated and amplified using Primers A and D to create the full VACChT\textsuperscript{G342R} sequence. Precipitation was carried out by adding 7.5M NH\textsubscript{4}OAc (Sigma-Aldrich, UK) at half the volume of product, and 1 volume of isopropanol (Fisher Scientific, UK) to the PCR product and incubating at room temperature for 25 mins. This was then washed with 70% ethanol (Fisher Scientific, UK), air dried and re-suspended in 20 µl ddH\textsubscript{2}O.

Initially the sequence was inserted into pBSII (PBluescript II KS; Stratagene Inc, USA) because it has a high copy number in \textit{E.coli}. \textit{E.coli} containing the plasmid was incubated for 16 hrs at 37°C, 250 rpm in LB broth (5 g NaCl, 10 g Tryptone, 5 g yeast extract in 1L water, autoclaved and 1 ml ampicillin (50 mg/ml stock, Melford, UK)). Plasmid was extracted using a QIAprep miniprep (QIAGEN). The PCR products for VACChT\textsuperscript{+} and VACChT\textsuperscript{G342R} were purified on a gel and bands which corresponded to the correct size (1.7kb) excised on a UV lightbox. DNA was extracted by adding 1 volume of 6M NaI (Sigma-Aldrich, UK) and heating to 55°C until the gel melted. 20 µl of glass milk (100 mg/ml silica in 3M NaI) was then added and the samples incubated for 5 minutes before being washed 3 times with New Wash solution (50% ethanol, 50 nM NaCl, 10 mM Tris-Cl pH7.5, 2.5 mM EDTA, pH8.0) and re-suspended in 20 µl TE buffer (10 mM Tris-Cl pH8.0, 1 mM EDTA, pH8.0). Both plasmid and purified PCR products were then digested with restriction enzymes Asc1 and Pac1 to create ‘sticky ends’. VACChT\textsuperscript{+} and VACChT\textsuperscript{G342R} were

**Figure 2.2** Schematic representing primer binding positions of primers A-D within the VACChT sequence

Primers A and D amplify the whole sequence. Primer C contained a nucleotide change at position 1025 which introduced the G342R substitution.
ligated with the plasmid in separate reactions using T4 DNA ligase (Fermentas, Thermo Fischer Scientific, UK) for 30 minutes at room temperature. The ligated plasmids were then inserted into CaCl$_2$ chemically competent E.coli by heat shock (42°C for 45 secs). Cells were plated onto agar plates (LB broth (inc. ampicillin) + 15 g agar) and stored at 37°C overnight before being transferred to 5°C. The pBSII plasmid contains a gene encoding ampicillin resistance so therefore only cells which contained the plasmid would be able to survive. Individual colonies were picked and incubated in LB broth for 16 hrs. Plasmid was then extracted and ran on 0.7% gel to check size, which was 4.7kb when the VACHT sequence was successfully inserted into plasmid. The plasmids were also sequenced using primers T7 and T3 by the University of Manchester sequencing facility to confirm the inserts carried the correct sequence.

In order to add the UAS sequence needed for GAL-UAS driven gene expression, the VACHT sequence was ligated into the P[acman]1-3-4 plasmid which has been modified to include the UAS sequence (Alves-Silva et al., 2012) and carries a chloramphenicol resistance gene. E.coli containing the P[acman]1-3-4 plasmid were incubated overnight (LB broth + 1 ml 25 µg/ml chloramphenicol (Duchefa Biochemie, The Netherlands)) and plasmid was extracted. The PBSII plasmids with 1) VACHT$^+$ and 2) VACHT$^{G342R}$ and P[acman]1-3-4 were digested using restriction enzymes AscI and PacI and run on a 0.7% gel. The 1.7kb (VACHT) fragment from PBSII 1) and 2) and the largest fragment of P[acman]1-2-3 were excised from the gel and purified. The VACHT$^+$ and VACHT$^{G342R}$ sequences were then independently ligated to the P[acman]1-3-4 plasmid. The plasmid was then inserted into competent cells by heat shock and cells were plated, incubated at 37°C overnight and colonies picked. These colonies were incubated, had plasmid extracted and sequenced to confirm the sequences were correct. Whole plasmid was then injected into embryos using the Drosophila embryo injection service provided by BestGene (USA). This service returns Drosophila larvae which contain the UAS insert over a balancer chromosome and flies were selected against the balancer to create a homozygous stock. This work was carried out with the help of Dr Wei-hsiang Lin (Baines group, Manchester).

2.7 Electron microscopy

Third-instar wall-climbing larvae were dissected in phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na$_2$HPO$_4$, 2 mM KH$_2$PO$_4$, pH 7.4). Two
protocols were trialed to find which gave the best contrast of vesicle membranes. In both protocols CNSs were fixed in 2.5% glutaraldehyde (Agar Scientific, UK) in 100 mM HEPES buffer, pH 7.2 for a minimum of 1 hr. In protocol 1, CNSs were then rinsed twice with ddH2O and post-fixed in 1% osmium tetroxide (Agar Scientific, UK) and 1.5% potassium ferrocyanide (Sigma-Aldrich, UK) in 0.1M cacodylate buffer for 1 hr, rinsed twice and then stained in aqueous 1% uranyl acetate (Chemistry, University of Manchester, UK) for 1 hr. A final two rinses were performed and CNSs were dehydrated in a graded ethanol and then a propylene oxide series and embedded in TAAB Low Viscosity resin (TAAB Laboratories Equipment Ltd). For protocol 2 each rinse stage was performed 5 times and after initial oxmium tetroxide staining (which was reduced to 30 mins), CNSs were incubated in 1% theocarbohydrazide (Sigma-Aldrich, UK) for 20 mins at room temperature before being transferred to 2% osmium tetroxide for a further 30 mins. The CNS was sectioned from 200 µm in from the posterior of the ventral nerve cord to try and include synapses to the dorsal motoneurons recorded in electrophysiological recordings. Images were taken from the anterior third of the neuropil. Sections were 70-80 nm thick and observed with a FEI Tecnai 12 Biotwin Transmission Electron Microscope. Sections were searched for synapses characterized by pre- and postsynaptic membranes with increased density of the membrane and clustering of vesicles. Electron micrographs were taken at 11,000X and random images were taken at 1900X magnification to assess number of active zones. CASPP treated larvae had been acutely fed compound for 24 hrs prior to dissection.

Images were coded and randomized for blind analysis. Images were analysed in ImageJ 1.47v (USA). Length of the pre-synaptic membrane was measured, the shortest distance from the centre of each vesicle to the membrane and the diameter of each vesicle. Only vesicles within 200 nm of the pre-synaptic membrane were included to avoid inclusion of vesicles closer to a neighboring synapse. In random images, taken at 1900X magnification, the ImageJ unbiased frame function was used to create a randomly placed frame of 29.4 µm² within the image. The number of active zones within each frame was counted, only frames which had vesicle containing tissue in >50% of their area were included in analysis. CNS sectioning was carried out by Dr. Aleksandr Mironov (Manchester) and images were taken with his assistance.
2.8 Electrophysiology

2.8.1 Larval

Dissections were carried out in ‘external’ saline (135 mM NaCl, 5 mM KCl, 4 mM MgCl₂-6H₂O, 2 mM CaCl₂, 5 mM TES, and 36 mM sucrose, at pH 7.15). The cuticle was gripped using fine forceps on the dorsal surface towards the anterior of the larvae and gently pulled apart, revealing the CNS. The mouth hooks were removed along with the gut. The CNS was then transferred to a coverslip coated in cured Sylgard Elastomer (Dow Corning, USA) and secured with GLUture topical tissue adhesive (World Precision Instruments, UK). The neurolemma covering the VNC was disrupted using a 1% w/v protease (bacterial protease type XIV, Sigma-Aldrich, UK) solution (in external saline) and was applied by positive pressure through a 10-20µm diameter borosilicate glass pipette.

Whole-cell patch-clamp recordings were carried out at room temperature (20-22°C) following the technique described by Marley and Baines (2011). Patch pipettes were pulled from borosilicate glass (GC100F-10, Harvard Apparatus, WPI, USA) using a P-97 Flaming/Brown micropipette puller (Sutter Instruments, USA) and heat-polished to a resistance of 8-12.5 MΩ. Recordings were performed using an Axopatch-1D amplifier (Axon instruments, USA) controlled by pClamp 10.4 and a Digidata 1440A (Molecular Devices, USA). Neurons were initially identified by GFP in Ra-GFP larvae, which express GFP in aCC and RP2 motoneurons in third-instar larvae (Fig. 2.2). After practice these neurons could be identified by their location, size and axonal projections without the need of GFP expression. In these recordings, AlexaFluor 488 (0.22 mM, Invitrogen) tip-filled pipettes were used to label neurons for unequivocal identification. Internal patch saline contained (140 mM K⁺ gluconate, 2 mM MgCl₂-6H₂O, 2 mM EGTA, 5 mM KCl, and 20 mM HEPES, pH 7.4). Only cells with an input resistance >400 MΩ were used for analysis. Cell capacitance was calculated in Clampfit 10.4 by integration of the area of the transient currents averaged for ten 30 mV steps from -60 mV to -90 mV. Chemicals were obtained from the following manufacturers: KCl from Fisher Scientific (UK), CaCl₂ from Fluka Analytical, Sigma-Aldrich (UK), NaCl, MgCl₂-6H₂O, TES, K⁺ gluconate, EGTA, HEPES from Sigma-Aldrich (UK).
Miniature synaptic currents (minis) were recorded by inclusion of 2 µM tetrodotoxin (TTX; Alomone labs, UK) in the external saline to block action potential induced spontaneous rhythmic currents (SRCs). Cells were held at -60 mV and recordings were sampled at 100 kHz with a lowpass filter of 0.5 kHz applied. Analysis of recordings, which were a minimum of 3 minutes duration, was carried out in MiniAnalysis (Synaptosoft, USA). Peaks were selected based on a fast rise time (time from baseline to peak) with a clear peak and a slower decay time (time from peak to return to baseline) and a minimum amplitude of 2 pA was set, representative of the size of the noise in recordings. Minis were also analysed in Clampfit 10.4, using the threshold search function to select all events above threshold amplitude, the user then individually accepts or rejects each event as a mini. Amplitude and area are calculated by the software and data was then transferred to Origin 9.0 (OriginLab Corporation, USA) for statistical analysis. For compound application recordings, larvae were acutely fed 5CL-CASPP, vesamicol or vehicle on grape agar (Genesee Scientific, USA) coated plates for ≥ 24 hrs before recording. For direct application to the CNS, the external saline was exchanged for saline containing 100µM vesamicol, control recordings contained vehicle (ethanol) in the external saline.

SRC recordings were sampled at 20 kHz with a lowpass filter of 0.2 kHz. Three minute duration recordings were analysed in Clampfit 10.4 (Molecular Devices, USA), the threshold search function was used to identify events over the threshold amplitude of 150 pA (set to avoid including large minis sometimes found when VACHT is overexpressed). Amplitude, area and inter-event interval are calculated by
the software. Duration was measured manually as the time from the steepest section of the rising slope to the point the decay slope returns to baseline. Recordings using bafilomycin A1 (Sigma-Aldrich, UK) were carried out by dissolving 10 µg bafilomycin A1 in 20 µl DMSO (Sigma-Aldrich, UK) before diluting to a 100 µM stock in external saline. This was added to the bath to a final concentration of 3 µM (0.4% DMSO). SRCs were recorded for 20 minutes from application and control recordings included 0.4% DMSO. The threshold search function of Clampfit 10.4 was used to analyse the recordings.

Raised temperature recordings were carried out using a heated perfusion tube (HPT-2A; ALA scientific Instruments, USA) regulated by a TC-10 temperature control system (npi electronics, Germany). This allowed heated saline to flow to a perfusion chamber (RC-26G, series 20; Warner Instruments, USA) mounted on a P-1 chamber platform (Warner Instruments, USA). Input was gravity fed and a peristaltic pump (323; Watson-Marlow, UK) removed saline out of the chamber. A temperature sensor was also placed in the recording chamber which formed a feedback loop to the temperature control system. CNSs were glued (GLUture) to Sylgard-coated 15 mm round coverslips (CS-15R; Warner Instruments, USA) and were placed into the perfusion chamber. A flow rate of 1.5 ml/min was used. Channelrhodopsin driven recordings were stimulated at 1 Hz using a 6002 stimulator (Harvard Apparatus, USA) to stimulate 2 ms flashes of blue light from an LED (bandwidth 25 nm, irradiance 466±14 nW·cm⁻²; OptoLED; Cairn Instruments, UK).

ACh response experiments were performed using the perfusion system described previously. 0.3 mM ACh (Sigma-Aldrich, UK) diluted in external saline was allowed to perfuse the chamber for 1 minute before being washed off. TTX (2 µM) was included in the external saline to prevent SRCs and amplitude of response was measure in Clampfit 10.4.

Current clamp recordings were sampled at 5 kHz and resting membrane potential was adjusted to -60 mV. Intrinsic membrane excitability was measured by injecting current in 4 pA steps. Number of action potentials induced at each step was counted by eye.
2.8.2 Adult GFS

Recordings from the giant fibre system (GFS) were carried out in adult flies (1-6 days old) using the technique described by Allen and Godenschwege (2010). Flies were anaesthetised by placing on ice for ~5 mins and mounted in white periphery wax (Kemdent, UK). The legs and wings were secured by pulling them laterally away from the body and pushing into the wax and the proboscis was gently pulled away from the head and secured by wax. The flies were mounted at a 45° angle to aid insertion of recording electrodes. For compound application, flies had either been treated in contact/feeding assays or were injected with compound containing GFS saline (101 mM NaCl, 1 mM CaCl₂, 4 mM MgCl₂, 3 mM KCl, 5 mM glucose, 1.25 mM NaH₂PO₄, 20.7 mM NaHCO₃, adjusted to pH 7.2) using a microinjector (Nanoliter 2010; World Precision Instruments, USA).

Flies were impaled through the abdomen with an earth electrode and then two sharpened tungsten stimulating electrodes were inserted, one through each eye, into the CNS where it sits at the back of the head capsule (Fig. 2.3B). For thoracic stimulation, the stimulating electrodes were pushed ~2-3 mm into the thorax behind the head. The earth and stimulating electrodes were made from 0.2 mm tungsten wire (Goodfellow, UK) which was electrolytically sharpened by attaching the tungsten electrode to the positive output of a stimulator (SD9; Grass Technologies, USA) and attaching a carbon rod to the negative output. Both the electrode and carbon rod were immersed in 4M NaOH and voltage output set to 10V. The tungsten could be sharpened by slowly inserting and removing it from the solution.

The stimulating electrodes were attached to a stimulus isolation unit (SIU5A; Grass Technologies, USA) which was driven by a stimulator (S88; Grass Technologies, USA). Initially a 0.02 ms pulse was applied while slowly increasing the voltage until a twitching of wings and/or TTM muscle could be seen by eye. Recording electrodes were pulled from borosilicate glass (GC100FS-10; Harvard Apparatus, WPI, USA) using a P-87 Flaming/Brown micropipette puller (Sutter Instruments, USA) to a resistance of 17 – 30 MΩ, filled with 3M KCl and inserted into the DLM muscle 45a and the TTM muscle on the contralateral side, identified by position of the bristles on the thorax (Fig. 2.3A, B). Both muscles lie just below the cuticle and therefore the electrodes should only be pushed just through the cuticle. Recordings were performed using an Axoclamp-2A amplifier (Axon instruments, USA) controlled by pClamp 10.4 and a Digidata 1440A (Molecular Devices, USA). Single pulses were applied and voltage was increased until a response in the muscles could be
reliable obtained, the voltage was then set to just above this threshold value (usually 40-60V).

**Figure 2.4** Position of TTM and DLM muscles in the adult fly

**A.** Location of DLM and TTM muscles identified by bristle positions. The TTM is can be located between the posterior and anterior supra-alar bristles. The DLM 45a muscles are located adjacent to the anterior dorso-central setae on the side of the midline. **B.** Position of electrodes during stimulation and recording from the GFS.

Response latency (time between stimulus and response) was tested by applying 5 single stimuli (0.02 ms) with a 5 sec interval between stimuli. Following frequency (ability to respond 1:1 with stimuli) was tested by applying 10 trains of 10 stimuli at 100 Hz with a 2 sec interval between trains. To assess the effect of injected
compounds, a train of 10 stimuli at 100 Hz was given every 30 seconds, from approximately 1 minute before injection. Data was sampled at 100 kHz. Recordings were analysed in Clampfit 10.4, response latency was measured as time from stimulus artefact to the onset of the muscle response and following frequency was calculated as successful responses out of 10.

2.9 Statistical analysis

Statistical analysis was carried out using a Student’s t-test to compare between two groups or one-way ANOVA when group size was ≥ 3. Analysis was carried out using Origin 9 software (Originlab, USA) or GraphPad Prism 6 software (GraphPad Software, USA). The post-hoc test applied after ANOVA was Bonferroni. In GFS analysis where there were unequal sample sizes the Scheffe post-hoc test was applied. Statistical significance denoted by asterisks is defined as the following p-values: ≤ 0.05 (*), ≤ 0.01 (**) and ≤ 0.001 (***).
Chapter 3

Investigating Mode of Action of Spiroindolines in *Drosophila* Larvae

3.1 Introduction

In insects the predominant excitatory neurotransmitter is ACh (Gauthier, 2010). The cholinergic synapse is therefore a prime target for insecticides. Current targets within the cholinergic synapse are shown in Fig. 3.1 and include: AChE, which catalyses the hydrolysis of ACh (Siman and Sussman, 2008), and nAChRs which mediate the postsynaptic response to ACh (Corringer *et al*., 2000). If AChE is inhibited, a build-up of ACh in the synaptic cleft leads to increased opening of nAChRs which then become desensitized, causing paralysis. Insecticides that target the nAChR can block or modulate activity of this receptor class in a competitive or allosteric manner. Insecticides with novel modes of action are constantly being sought because persistent use of insecticides with the same mode of action leads to a selection pressure to induce development of resistance. Recently, compounds that have a novel target protein within the cholinergic synapse have been identified. In particular, the spiroindoline compounds, developed by Syngenta (Sluder *et al*., 2012), inhibit the VAChT (Fig. 3.1). The VAChT loads ACh into SVs at the presynapse. However, although it is known that the spiroindolines bind to the VAChT, their effect on ACh release at central cholinergic synapses has not been demonstrated.

Previously, the only compound known to block the VAChT was vesamicol (Khare *et al*., 2010). Although highly toxic to mammals (Brittain *et al*., 1969) it is not insecticidal and shows low affinity binding in insect tissue (Sluder *et al*., 2012). At the snake NMJ, vesamicol causes a decrease in single SV release frequency but not amplitude of miniature endplate currents (mEPC) (Parsons *et al*., 1999). In rat motor endplates, vesamicol treatment results in a population of mEPCs with decreased amplitude following nerve stimulation (10 Hz, 5 min) (Searl *et al*., 1991) and reduced amplitude of mEPCs is reported at frog NMJ (van der Kloot, 2000). Similarly, SVs at the mouse NMJ are reduced in size but not number after being
treated with vesamicol, however this study does not account for empty SVs (Rodriguez et al., 2013). Based on this prior evidence, it might be expected that spiroindolines would cause a reduction in ACh release at the cholinergic synapse through decreasing availability of ACh containing SVs. This chapter explores the effect of a spiroindoline compound (5Cl-CASPP) on Drosophila larval mortality, behaviour and cholinergic input to identified motoneurons (aCC/RP2). The ability to perform whole-cell patch-clamp from identified motoneurons is one of the benefits of using Drosophila as a model to study insecticide mode of action. The capacity to easily manipulate gene expression and a relatively simply genome also make Drosophila an attractive model.

![Figure 3.1](image)

**Figure 3.1** Insecticide targets within the cholinergic synapse

1. The nAChR located in the membrane of the postsynaptic cell. 2. The AChE enzyme found bound to the postsynaptic membrane close the nAChR and 3. The VACHT located in the membrane of SVs at the presynapse.

Cholinergic synapses between cholinergic premotor interneurons and the aCC motoneuron present a powerful candidate model to explore the effect of manipulation of VACHT activity. The aCC neuron can be identified by location and characteristic dendritic branching in the CNS and by axonal extension to innervate muscle 1 in the larval body wall (Landgraf et al., 1997). A lot is known about the synaptic input, ionic currents and transcription factors that affect cell function of this neuron (Baines et al., 1999, 2002; Baines, 2003; Mee et al., 2004; Garces and Thor, 2006; Pym et al., 2006; Lin et al., 2009; Wolfram et al., 2012, 2014; Günay et al., 2015). Work is still ongoing to better characterise this motoneuron, including identifying the interneurons that synapse with it (Couton et al., 2015). This chapter also aims to contribute to the knowledge known about this motoneuron by
examining the subunit composition of the nAChRs, which mediate the cells response to ACh. This knowledge will also improve this synapse as a model for insecticide mode of action research as specific subunits may respond differently to the same insecticide.

The nAChR is a pentameric channel (Itier and Bertrand, 2001) comprised of 5 subunits from a possible 10 found in *Drosophila* (Dα1-7, Dβ1-3) (Jones and Sattelle, 2010): the precise composition of subunits effects how the receptor responds to ACh. For example, an increase in the Dα7 subunit has been shown to increase amplitude of miniature endplate synaptic currents (Ping and Tsunoda, 2012). Fluorescent activated cell sorting and molecular biology will be used to elucidate the composition of the nAChRs in aCC motoneurons of third-instar larvae.

### 3.2 Results

The effect of spiroindolines on *Drosophila* was initially assessed by mortality assays in larvae. This was also undertaken to identify suitable doses to use for functional studies. Transgenic flies were used to manipulate the VAChT and assess change in mortality. Crawling behaviour was assessed to examine the effect on speed and distance travelled. It is already known that the spiroindolines block the VAChT (Sluder *et al.*, 2012) but the effect this has on ACh release at the synapse has not been demonstrated. The specific effect of the spiroindoline 5Cl-CASPP on the cholinergic synapse was examined by patch-clamp recording from postsynaptic motoneurons in third-instar wall-crawling larvae. Both the action potential independent random release of single SVs (minis, quanta) and action potential-dependent release of ACh as spontaneous rhythmic currents (SRCs, multiple SV release) were measured. The subunit composition of the postsynaptic nAChR was evaluated using PCR and response to ACh.

#### 3.2.1 CASPP induced mortality in *Drosophila* larvae

To assess lethality of spiroindolines to larvae and find concentrations appropriate to use in later electrophysiological experiments, mortality assays were carried out. Two spiroindoline compounds were initially tested (5F-CASPP and 5Cl-CASPP) on
larvae (genotype: RRa-GFP) in chronic feeding assays as described in chapter 2. When mortality was compared at each concentration, no significant difference between the two compounds was observed (P > 0.05 at all concentrations, Fig. 3.2A). It was therefore decided that only one of the compounds would be taken forward for further experiments. 5F-CASPP gave higher variability in mortality (as illustrated by large error bars) and did not show a typical dose response, therefore 5Cl-CASPP was chosen.

5Cl-CASPP (from here on referred to as CASPP) was tested on the following larval genotypes: CS (wild-type), cha>VACHT<sup>+(SYN)</sup> (wild-type VACHT overexpressed in cholinergic neurons), cha>VACHT<sub>Y49N</sub> (CASPP resistant VACHT) and their parental strains (Fig. 3.2B). The UAS_VACHT<sup>+</sup> transgenic fly line used in this chapter is referred to as VACHT<sup>+(SYN)</sup> for purposes of discrimination from a similar transgenic line created during the course of this work. VACHT<sup>+(SYN)</sup> was provided by Syngenta and subsequent sequencing showed it to have a polymorphism equating to a missing a glutamine residue in a polyQ domain at position 549. The differences in this polymorphism variant of VACHT are discussed in more detail in chapter 4. LD<sub>50</sub> values indicating the dose estimated to cause 50% mortality are shown in Table 3.1. Cha>VACHT<sup>+(SYN)</sup> shows a ≥ 3-fold decrease in sensitivity to CASPP and cha>VACHT<sub>Y49N</sub> larvae were insensitive to all concentrations of CASPP tested. This is in agreement with previously published data (Sluder <i>et al.</i>, 2012).

Cha>VACHT<sup>+(SYN)</sup> and cha>VACHT<sub>Y49N</sub> were significantly different from parental controls at 2 µg/ml (P < 0.05) and 4-10 µg/ml (P < 0.001 in all cases). This demonstrates that susceptibility to CASPP can be reduced through either increased expression of the target protein and/or via a point mutation. It was unexpected that transgenic parental controls would be more sensitive to spiroindoline than wild-type (CS) or RRa-GFP and this may be due to differences in the genetic background of these strains. The mechanism behind the resistance conveyed by this point mutation is currently unknown.

Concentrations from mortality assays were chosen for further experiments. The LD<sub>100</sub> concentration that gave ~100% mortality in controls (10 µg/ml) and the LD<sub>50</sub> dose (5 µg/ml) were used. For the former, escapers were used for recordings. From here forward these doses will be referred to as the quantity of compound applied. Thus the LD<sub>100</sub> consisting of 50 µl of 10 µg/ml equates to a final amount of 0.5 µg. The LD<sub>50</sub> consisting of 50 µl of 5 µg/ml is 0.25 µg in total amount. This is to avoid confusion between mortality assays and acute compound exposure (see methods).
Figure 3.2 CASPP compounds do not differ in potency and mortality is reduced when wild-type or resistant VACHT is overexpressed

A. CASPP compounds 5Cl-CASPP and 5F-CASPP do not show a difference in potency in larval survival assays. B. Mortality is significantly reduced in cha>VACHT+(SYN) and cha>VACHTY49N larvae. Significance represented by asterisks is between parental controls (cha and UAS) and cha>VACHT+(SYN) (top) and cha>VACHTY49N (bottom). Average values are derived from 3 independent samples of 20 larvae. Values are mean ± SEM.
3.2.2 VAChT dependence of larval locomotion

CASPP has been shown to bind the VAChT and is predicted to affect ACh loading into SVs thereby reducing the availability of loaded SVs at the cholinergic synapse. Motoneurons which are postsynaptic to cholinergic interneurons in *Drosophila* larvae are glutamatergic and synapse onto the body wall muscles, directing locomotion. It may therefore be expected that CASPP would have a downstream effect on larval locomotion. To assess this, larval tracking assays, using third-instar wall crawling larvae, which had been acutely fed the highest tolerable dose of CASPP for 24 hrs (0.5 µg) were carried out. CASPP was shown to reduce both speed of crawling and distance travelled in exposed wild-type larvae (distance: 7.7 ± 0.03 vs. 9.8 ± 0.5 cm, P = 0.005; speed: 3.9 ± 0.2 vs. 5.0 ± 0.3 cm per min, P = 0.009, 0.5 µg CASPP vs. control, Fig. 3.3). Because blocking the VAChT negatively impacts on locomotion, it might be predicted that overexpressing the VAChT would increase speed and/or distance. Therefore, tracking assays were also carried out on *cha>*VAChT+(SYN) larvae to assess if increased VAChT could affect larval crawling.

No significant difference was seen compared to control (combination of *cha* and *cha*+) for either distance travelled or speed (distance: 6.4 ± 0.7 vs. 7.9 ± 0.4 cm, P = 0.1, speed: 3.3 ± 0.3 vs. 4.0 ± 0.2 cm per min, P = 0.2, *cha>*VAChT+(SYN) vs. control). It is often reported that mutations which convey resistance to a pesticide also confer a fitness cost (Kilot and Ghanim, 2012). To assess if there was a cost to

### Table 3.1 Overexpressing VAChT reduces sensitivity to CASPP.

The dose of CASPP required to induce 50% mortality in *cha>*VAChT+(SYN) larvae is ~ 3-fold higher than controls. RRa-GFP expresses GFP in aCC and RP2 neurons. Cha, UAS-VAChT+(SYN) and UAS-VAChT+Y49N are transgenic GAL4 and UAS parental controls. *Cha>*VAChT+(SYN) expresses wild-type VAChT in cholinergic neurons. Values are mean LD50 ± SEM.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>CASPP</th>
<th>LD50 (µg/ml, n = 3)</th>
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<tbody>
<tr>
<td>RRa-GFP</td>
<td>5F-CASPP</td>
<td>5.5 ± 1.2</td>
</tr>
<tr>
<td>RRa-GFP</td>
<td>5Cl-CASPP</td>
<td>5.8 ± 0.3</td>
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<tr>
<td>CS (wild-type)</td>
<td>5Cl-CASPP</td>
<td>5.6 ± 0.1</td>
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<tr>
<td>cha</td>
<td>5Cl-CASPP</td>
<td>2.6 ± 0.2</td>
</tr>
<tr>
<td>UAS-VAChT+(SYN)</td>
<td>5Cl-CASPP</td>
<td>3.5 ± 0.03</td>
</tr>
<tr>
<td>UAS-VAChT+Y49N</td>
<td>5Cl-CASPP</td>
<td>3.4 ± 0.4</td>
</tr>
<tr>
<td>*cha&gt;*VAChT+(SYN)</td>
<td>5Cl-CASPP</td>
<td>13.4 ± 1.7</td>
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</table>
locomotion in *Drosophila* of the Y49N mutation, *cha>*VAChT<sup>Y49N</sup> larvae were also tracked. Again no difference in distance travelled or speed was observed (distance: 9.0 ± 0.6 cm, *P* = 0.4; speed: 4.6 ± 0.2 cm per min, *P* = 0.4, *cha>*VAChT<sup>Y49N</sup> vs. control). It must be noted that endogenous wild-type VACHT is also present in these larvae and may be enough to compensate for any negative impact of VACHT<sup>Y49N</sup>. Some caution must be applied when interpreting these results as the sample size is relatively low when compared to other behavioural studies.

Fig. 3.3

**Figure 3.3** Crawling speed and distance travelled is decreased in CASPP treated larvae
A. Peak speed was significantly reduced in CASPP-fed larvae, but was not altered in cha\textgreater VAChT^{SYN} or cha\textgreater VAChT^{Y49N} larvae. B. Total distance travelled in 2 minutes was significantly reduced in CASPP-fed larvae, but again, was not affected in cha\textgreater VAChT^{SYN} or cha\textgreater VAChT^{Y49N} larvae. Values are mean ± SEM.

3.2.3 Motoneuron identification and validation of mini analysis

To assess the effect of CASPP on action potential independent quantal ACh release (release of single SVs, termed minis), whole-cell patch recordings were carried out from the aCC/RP2 motoneurons in third-instar larvae. Confirmation of the identity of motoneurons was enabled either by using RRa-GFP background (this line expresses GFP in aCC and, to a lesser extent, RP2) and/or by inclusion of the dye AlexaFluor\textsuperscript{488} in the recording patch electrode. To ensure that the inclusion of dye had no effect on current amplitude and frequency of minis, cells recorded with and without dye were compared (Fig. 3.4). Minis were selected for analysis based on their characteristic shape of a sharp inward current followed by a slower return to baseline (Fig. 3.4A). These currents were blocked in the presence of mecamylamine (nAChR antagonist) proving that they are cholinergic events (Fig. 3.4B). The inclusion of dye in the recording pipette did not affect amplitude or frequency of minis (amplitude: 6.8 ± 0.7 vs. 5.7 ± 0.4 pA, \(P = 0.2\), frequency: 55.5 ± 11.3 vs. 40.0 ± 6.9 per min, \(P = 0.3\), dye vs. no dye, Fig. 3.4B-C). It was therefore concluded that inclusion of dye had no effect on recordings.

Initially, two different software programs for analysis were trialled. The first software was MiniAnalysis in which mini events are individually selected by the user, whilst the second software package Clampfit 10.4 utilised an automated threshold search function. The values from each analysis were compared and found to show no difference in amplitude or frequency (amplitude: 5.8 ± 0.2 vs. 5.7 ± 0.4 pA, \(P = 0.8\), frequency: 39.3 ± 7.5 vs. 40 ± 6.9 per min, \(P = 0.9\), Clampfit vs. MiniAnalysis). The threshold search function in Clampfit was more prone to errors as it selected all events over the 2 pA threshold which often included a lot of events which were noise (requiring visual inspection to delete those events). It was therefore decided to use MiniAnalysis to analyse all mini recordings because this method is quicker and less prone to false positives.
**Fig. 3.4**

A. Mini identification

Accept vs. Reject

5 pA
20 msec

B. RRa-GFP

RRa-GFP + mecamylamine (1 mM)

Dye vs. no dye
Clampfit vs. MiniAnalysis

C. Amplitude

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<th>+ dye</th>
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D. Frequency

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E. Amplitude

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F. Frequency

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<td>n = 8</td>
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Figure 3.4  Inclusion of dye in the recording pipette does not affect mini recordings and analysis is not affected by choice of software

A. Traces representing a variety of typical minis which would be accepted and those that would be rejected as noise or not representative of single SV release. B. Traces representing a control mini recording and a recording where nAChR is blocked by mecamylamine. C/D. Fluorescent dye (AlexaFluor<sup>488</sup>) does not affect mini amplitude or frequency. E/F. The measurement of mean mini amplitude and frequency is not different in Clampfit or MiniAnalysis. N represents number of neurons recorded, each for a 3 min period, and not number of events. For each neuron recorded an average of event amplitude and frequency was calculated and these were then used to provide the data shown. Values are mean ± SEM.

3.2.4 The effect of CASPP on quantal release of ACh at cholinergic synapses

It has been demonstrated that CASPP causes mortality in <i>Drosophila</i> larvae and has a downstream effect on locomotion. It is therefore probable that blocking the VACChT has a direct effect on ACh released at cholinergic synapses. To assess this, minis were recorded by blocking all action potential-dependent release using TTX in the extracellular saline (see methods). Recordings were made from RRa-GFP larvae that were acutely fed either 0.5 µg CASPP or vehicle for 24 hours from early third-instar larvae (Fig. 3.5). Exposure to CASPP decreased the probability of release (frequency) but did not affect amplitude (frequency: 6.5 ± 0.6 vs. 16.2 ± 2.7 per min, <i>P</i> = 0.001; amplitude: 6.4 ± 0.3 vs. 6.7 ± 0.6 pA, <i>P</i> = 1.0, 0.5 µg CASPP vs. control, Fig. 3.6A,B). Cumulative probability plots show that there is no shift in amplitude of the population of minis, further confirming no effect and also showing no existence of a sub-population of smaller minis (Fig. 3.6C). The effect on frequency was dose dependent as shown by recordings at 0.25 µg which show no effect (frequency: 10.9 ± 1.5, <i>P</i> = 0.2, amplitude: 6.5 ± 0.2, <i>P</i> = 1.0, 0.25 µg CASPP vs. control). These results suggest that CASPP causes mortality by reducing the availability and/or probability of release of loaded cholinergic SVs at the synapse. It may also demonstrate that at this synapse, SVs only contain one VACChT as no minis with a reduced amplitude were recorded.

Minis were recorded from <i>cha>-VACChT<sup>T<SUP>+</SUP>SYN</i> larvae to examine the effect of increased levels of VACChT on quantal release of ACh. Where GFP was not present,
dye was used in the recording pipette to verify the motoneuron post-recording. Larvae which overexpressed this wild-type form of the VAChT showed a doubling in mini frequency compared to controls (combined RRa-GFP, cha/+,
UAS_VAChT^{(SYN)}/+ and UAS_VAChT^{Y49N}/+) and an increase in amplitude (frequency: 98.2 ± 10.5 vs. 41.7 ± 4.9 per min, P < 0.001; amplitude: 10.7 ± 0.7 vs. 7.2 ± 0.2 pA, P < 0.001, cha>VAChT^{(SYN)} vs. control, Fig. 3.5 and 3.6). Cumulative probability plots show that the whole population of cha>VAChT^{(SYN)} minis are higher in amplitude, suggesting that all released SVs contain more neurotransmitter (Fig. 3.6C). Minis were also recorded from cha>VAChT^{(SYN)} larvae which had been fed 0.5 µg CASPP acutely for 24 hours. Recordings in these larvae showed an expected reduction in frequency towards control levels (frequency: 33.5 ± 4.8 per min, P < 0.001, amplitude: 8.9 ± 1.1 pA, P = 0.3 vs. unfed cha>VAChT^{(SYN)}).
Cha>VAChT^{(SYN)} treated with CASPP were no longer different to parental controls in frequency or amplitude (frequency: P = 1.0, amplitude: P = 0.3). These results are consistent with CASPP acting to block the VAChT.

It would be expected that cha>VAChT^{Y49N} larvae would be resistant to the effect of CASPP on mini frequency. Unfed cha>VAChT^{Y49N} larvae showed no significant change in mini frequency or amplitude compared to control (frequency: 60.9 ± 7.2 per min, P = 0.8; amplitude: 7.8 ± 0.2 pA, P = 1.0, cha>VAChT^{Y49N} vs. control, Fig. 3.5 and 3.6). However, larvae which were acutely fed CASPP unexpectedly showed an increase in frequency that although not significantly different to unfed cha>VAChT^{Y49N} (frequency: 91.8 ± 9.7 per min, P = 0.2), was significantly different to parental controls (P < 0.001). Amplitude did not appear affected (7.0 ± 0.2 pA, P = 1.0 vs. control or untreated cha>VAChT^{Y49N}). Cumulative probability plots show no obvious shift in mini amplitude compared to control fed or unfed (Fig. 3.6C).

Taken together, my results suggest that overexpression of VAChT^{(SYN)} increases both amplitude and frequency of minis indicative of increased ACh content per SV and increased probability of release. Overexpression of VAChT^{Y49N} in the presence of CASPP (which presumably blocks the endogenous VAChT) increases further probability of SV release. It is possible that this mutation has functional consequences in addition to being insensitive to block by this insecticide.
Figure 3.5 Representative traces of mini recordings

A/B. Traces representing control larvae (RRa-GFP) and control acutely fed CASPP. C/D. Traces representing cha>VACht+(SYN) minis and cha>VACht+(SYN) fed CASPP. E/F. Traces representing cha>VAChtY49N minis and cha>VAChtY49N fed CASPP. An expanded region of each trace, with all minis marked by •, is shown on the right.
Fig. 3.6

**Amplitude**

<table>
<thead>
<tr>
<th>Condition</th>
<th>n</th>
<th>Amplitude (pA)</th>
<th>n.s.</th>
<th>p.A.</th>
</tr>
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<tbody>
<tr>
<td>RRa-GFP</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>RRa-GFP + 0.5μg</td>
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<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RRa-GFP + 0.25μg</td>
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<td>cha&gt;VAChT+/Y49N</td>
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<tr>
<td>cha&gt;VAChT+/Y49N + 0.25μg</td>
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**Frequency**

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<th>p.A.</th>
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<tr>
<td>RRa-GFP + 0.25μg</td>
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<td>18</td>
<td>50</td>
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</tbody>
</table>

**Cumulative probability of amplitudes**

- **Control**
  - RRa-GFP
  - RRa-GFP + 0.5μg

- **cha>VAChT+/SYN**
  - RRa-GFP
  - RRa-GFP + 0.5μg

- **cha>VAChT+/Y49N**
  - RRa-GFP
  - RRa-GFP + 0.5μg
Figure 3.6  Amplitude and frequency of minis in different genotypes acutely fed CASPP

A. Mini amplitude was unchanged in CASPP-fed RRa-GFP larvae. Cha>VACHT<sup>+</sup><sup>(SYN)</sup> larvae showed a significant increase in amplitude which was reduced on CASPP feeding. Cha>VACHT<sup>Y49N</sup> larvae did not show a significant increase in amplitude and was also not affected by CASPP. B. Frequency was significantly decreased in RRa-GFP larvae fed CASPP. Cha>VACHT<sup>+</sup><sup>(SYN)</sup> larvae showed a significant increase in frequency which was decreased in CASPP-fed larvae. Cha>VACHT<sup>Y49N</sup> larvae did not show a significant increase in frequency but those fed CASPP did. Cha>VACHT<sup>+</sup><sup>(SYN)</sup> and cha>VACHT<sup>Y49N</sup> are combined recordings from different time points. Control for cha>VACHT<sup>+</sup><sup>(SYN)</sup> and cha>VACHT<sup>Y49N</sup> was combined RRa-GFP, cha<sup>+</sup>, UAS_VACHT<sup>+</sup><sup>(SYN)</sup>/+ and UAS_VACHT<sup>Y49N</sup>/+. C. Cumulative probability of amplitudes is unchanged in CASPP treated larvae. Cha>VACHT<sup>+</sup><sup>(SYN)</sup> larval minis show a shift to higher amplitudes which shifts back in CASPP-fed larvae. Cha>VACHT<sup>Y49N</sup> larvae do not show a shift in cumulative probability of amplitudes. Values are mean ± SEM.

3.2.5 Alternative methods to reduce VACHT activity

CASPP causes a decrease in frequency but not amplitude of minis. To show that this was directly caused by an effect on the VACHT, other methods to block or reduce the VACHT were used. The first of these to be tested was vesamicol, which was initially tested in larval mortality assays and was shown to not cause increased mortality at concentrations tested up to 1000 µg/ml (total quantity = 50 µg) (P = 0.2 vs. control, Fig. 3.7A). This is not surprising given that vesamicol is selective for vertebrate VACHT (Sluder et al., 2012). However, to investigate if there was still a sub-lethal effect on ACh release, mini recordings were carried out in RRa-GFP larvae acutely fed 50 µg vesamicol for 24 hours (Fig. 3.7B). No significant difference to control was observed for amplitude or frequency of minis (amplitude: 6.8 ± 0.6 vs. 8.6 ± 0.8 pA, P = 0.1, frequency: 18.8 ± 5.7 vs. 16.1 ± 2.3 per min, P = 0.7, vesamicol vs. control). Because vesamicol is a compound known to bind VACHT it would be the ideal comparison to CASPP. I therefore also tried applying vesamicol to the external saline before recording from motoneurons, because the lack of mortality in assays may be due to vesamicol being unable to access the target. Isolated CNSs were incubated for a minimum of 15 (and a maximum of 25) mins prior to recording (later times are when more than one cell was recorded per CNS). Vesamicol (100 µM) caused a significant decrease in mini amplitude and frequency (amplitude: 5.0 ± 0.2 vs. 6.3 ± 0.4 pA, P = 0.008, frequency: 1.3 ± 0.2 vs. 12.3 ± 3.0.
per min, \( P = 0.003 \), vesamicol vs. control, Fig. 3.7). This supports the findings with CASPP that blocking VAChT causes a decrease in mini frequency but also suggests that vesamicol acts in a different way to CASPP to reduce mini amplitude.

To try and replicate the effect of CASPP genetically, a UAS\_VAChT^{RNAi} line was overexpressed in cholinergic neurons. This produced no effect on mini amplitude or frequency (amplitude: \( 8.3 \pm 0.3 \) vs. \( 8.0 \pm 0.8 \) pA, \( P = 0.7 \), frequency: \( 34.0 \pm 7.8 \) vs. \( 41.3 \pm 3.9 \) per min, \( P = 0.5 \), cha\_VAChT^{RNAi} vs. control, Fig. 3.7B-C). One possibility is that the RNAi line was not able to sufficiently deplete VAChT transcript to cause an effect (efficacy was not tested by PCR). Recordings were also carried out in the VAChT\(^1\) heterozygous mutant (i.e. +/-) (Kitamoto et al., 2000), where one copy of the VAChT gene is non-functional. I again saw no effect on mini properties (amplitude: \( 6.3 \pm 0.5 \) vs. \( 7.8 \pm 0.6 \) pA, \( P = 0.1 \), frequency: \( 30.1 \pm 6.4 \) vs. \( 40.9 \pm 5.1 \) per min, \( P = 0.2 \), VAChT\(^1\)/TM6B vs. +/-TM6B). This suggests that one copy of the VAChT gene is sufficient for normal synaptic function.

Of the methods I have used, only acute application of vesamicol was able to mirror the reduction in mini frequency seen in CASPP treated larvae, supporting the hypothesis that it is caused by a direct effect on the VAChT. However, vesamicol also affected mini amplitude, indicating that vesamicol may have a second mode of action or does not completely prevent ACh loading into SVs.
Fig. 3.7

A  L(-)-vesamicol larval mortality

![Graph showing mortality percentage against concentration (µg/ml)]

B  Amplitude

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Amplitude (pA)</th>
<th>n</th>
</tr>
</thead>
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</tr>
<tr>
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<td>6</td>
</tr>
<tr>
<td>CNS application control</td>
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<td>12</td>
</tr>
<tr>
<td>CNS application 100µM</td>
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<td>12</td>
</tr>
<tr>
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C  Frequency

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</thead>
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<tr>
<td>VACHT/TM6B</td>
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Figure 3.7  Mortality in larvae fed with L(-)-vesamicol and the effect on minis of treatments aimed to replicate CASPP

A. Chronic feeding of vesamicol did not cause increased mortality at any concentration tested. B. Amplitude was not significantly changed in larvae fed vesamicol (control is RRa-GFP) but was significantly reduced when vesamicol was applied directly to CNS. Amplitude was not changed in cha>VAChT\textsuperscript{RNAi} (control is cha/+ and UAS_VAChT\textsuperscript{RNAi}/+) larvae nor null_VAChT\textsuperscript{1}/TM6C larvae (control is +/-TM6C). C. Frequency was not significantly changed in larvae fed vesamicol but was significantly reduced when vesamicol was applied directly to CNS. Frequency was not changed in cha>VAChT\textsuperscript{RNAi} larvae nor null_VAChT/TM6C larvae. Values are mean ± SEM.

3.2.6 The effect of manipulating the VAChT on spontaneous rhythmic currents

Spontaneous rhythmic currents (SRCs) are action potential-dependent events which are the result of many SVs being simultaneously exocytosed and can induce action potential firing in the postsynaptic cell. Therefore, any effect on these events would be predicted to be more critical for larval behaviour. When CASPP is acutely fed to larvae at 0.5 $\mu$g, reducing VAChT activity, no SRCs were recorded in ~50% of recordings (Fig. 3.8A,B). The other recordings showed SRCs which were greatly reduced in frequency, but there was no significant change to amplitude or duration compared to control (frequency: $1.5 \pm 0.5$ vs. $20.6 \pm 2.8$ per min, $P < 0.001$, current density: $21.1 \pm 3.8$ vs. $39.0 \pm 7.2$ pA/pF, $P = 0.1$, duration: $0.7 \pm 0.02$ vs. $0.7 \pm 0.04$ sec, $P = 1.0$, 0.5 $\mu$g CASPP vs. control, Fig. 3.8F-H). Amplitude is expressed in terms of current density: averaged SRC amplitude recorded in each neuron is divided by the cell capacitance. This normalises for the fact that bigger cells show bigger SRC currents. To determine if there was a concentration threshold for the loss of SRCs, a lower concentration of 0.45 $\mu$g was tested. SRCs recorded at this dose were not significantly different to control in current density, duration or frequency (current density: $41.1 \pm 5.7$ pA/pF, $P = 1.0$, duration: $0.8 \pm 0.02$ sec, $P = 0.2$, frequency: $15.5 \pm 2.1$ per min, $P = 0.3$, Fig. 3.8C,F-H). This surprising result suggests that the effect of CASPP occurs over a very short dose-range and is either ineffective or fully-effective in this preparation. This contrasts the effect of this compound on larval mortality, which shows a traditional dose-response.
By contrast to CASPP exposure, overexpression of VACHT caused a marked increase in SRC duration without a statistical effect on amplitude (duration: $1.4 \pm 0.2$ vs. $0.7 \pm 0.03$ sec, $P = 0.001$, current density: $50.9 \pm 6.5$ vs. $33.6 \pm 4.7$ pA/pF, $P = 0.1$, cha>VAChT^{(+SYN)} vs. control, Fig.3.8D-E). Frequency was not significantly changed ($18.0 \pm 2.5$ vs. $26.4 \pm 3.2$ per min, $P = 0.1$, cha>VAChT^{(+SYN)} vs. control, Fig.3.8H). The effect on duration was blocked by the co-feeding of CASPP (0.5ug) (Fig. 3.8E). This indicates that the effects on SRCs in cha>VAChT^{(+SYN)} larvae are due to changes in the presynaptic release of ACh.

Detailed observation of cha>VAChT^{(+SYN)} SRC recordings appeared to show that very large SRC events would be followed by a longer inter-event interval before the next event. This was tested by plotting instantaneous frequency of events against amplitude in the cha>VAChT^{(+SYN)} background (Fig. 3.8I). The correlation between amplitude and instantaneous frequency was tested using a linear regression, Pearson’s $r$ test that showed a significant correlation ($r = -0.29$, R-squared = 0.08, $P < 0.001$). The slope was also shown to be significantly different to 0 ($P < 0.001$). This is suggestive of a possible feedback mechanism from the postsynaptic motoneuron to the presynaptic interneurons which suppresses SV release after a large amplitude event.
Fig. 3.8

A) Control
B) Control + 0.5 μg
C) Control + 0.45 μg
D) cha-VACHT+(SYN)
E) cha-VACHT+(SYN) + 0.5 μg

Current density

<table>
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<tr>
<th>Condition</th>
<th>Current density (pA/pF)</th>
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<td>RRa-GFP + 0.5 μg</td>
<td>7</td>
</tr>
<tr>
<td>cha-VACHT+(SYN)</td>
<td>15 ± 2</td>
</tr>
<tr>
<td>cha-VACHT+(SYN) + 0.5 μg</td>
<td>17 ± 3</td>
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</table>

Duration

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<td>7</td>
</tr>
<tr>
<td>cha-VACHT+(SYN)</td>
<td>15 ± 2</td>
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<tr>
<td>cha-VACHT+(SYN) + 0.5 μg</td>
<td>17 ± 3</td>
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Frequency

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<td>cha-VACHT+(SYN)</td>
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Instantaneous frequency

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Amplitude

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<td>RRa-GFP + 0.45 μg</td>
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<td>RRa-GFP + 0.5 μg</td>
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<td>cha-VACHT+(SYN)</td>
<td>800 ± 80</td>
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<td>cha-VACHT+(SYN) + 0.5 μg</td>
<td>1000 ± 100</td>
</tr>
</tbody>
</table>

n.s: not significant

* * *: significant at p < 0.001
Figure 3.8  Representative SRC traces and analysis

A. Trace representing control SRCs (RRa-GFP). B. Traces representing SRCs from RRa-GFP larvae acutely fed 0.5 µg CASPP illustrating that no (1) or very few (2) events were recorded. C. Trace representing SRCs from RRa-GFP larvae acutely fed 0.45 µg CASPP. D. Trace representing SRCs from cha>VACHT+(SYN). E. Representative trace from cha>VACHT+(SYN) larvae fed CASPP. F. SRC amplitude (current density) was not significantly different in 0.45 or 0.5 µg CASPP-fed larvae, in cha>VACHT+ larvae or those fed CASPP. G. SRC duration was not changed in 0.45 or 0.5 µg CASPP-fed larvae. Duration was significantly increased in cha>VACHT+(SYN) larvae and decreased after CASPP feeding. H. SRC frequency was unchanged in 0.45 µg CASPP-fed larvae but was significantly decreased in 0.5 µg treated larvae. Frequency was unchanged in cha>VACHT+(SYN) and those fed CASPP. I. In cha>VACHT+(SYN) larvae instantaneous frequency increases with amplitude of event. Method of measuring instantaneous frequency is demonstrated by trace, distance from event to succeeding event was measured and correlated to amplitude of event. Cha>VACHT+(SYN) is combined recordings from 2 time points, control is RRa-GFP, cha/+ and UAS/+.

Value within bars represents N. Values are mean ± SEM.

3.2.7 Altering levels of active VACHT does not affect the postsynaptic response to ACh

The effects of manipulating VACHT appear to be presynaptic, but to determine if manipulating this transporter evokes postsynaptic compensation, specifically change in level of nAChR expression, I directly exposed the aCC motoneuron to applied ACh. aCC alone was used in this experiment as I was able to successfully identify this neuron more frequently than RP2 without the presence of GFP. It may be expected that decreasing or increasing VACHT would cause a homeostatic response to decrease or increase the response to ACh. This could be achieved by altering which subunits are incorporated into the nicotinic acetylcholine receptor (nAChR) or changing the number of receptors expressed at the postsynaptic site.

Initially, different concentrations of ACh were applied through perfusion (0.1, 0.3 and 0.5 mM) and it was found that 0.3 mM gave the largest and most consistent response (Fig. 3.9A). The decision to apply ACh through perfusion rather than iontophoresis was made because the exact location of the cholinergic synapses on this neuron are unknown and previous attempts to test ACh response by iontophoresis gave highly-variable results (Richard Baines, personal
communication). The amplitude of the response was measured and divided by cell capacitance to compensate for the size of the cell. Blocking VAChT with CASPP did not affect the response of aCC motoneurons to applied ACh (11.2 ± 2.0 vs. 12.2 ± 1.7 pA/pF, P = 0.7, CASPP vs. control, Fig. 3.9B). Overexpressing the VAChT also did not affect postsynaptic response to ACh (10.2 ± 2.9 vs. 8.3 ± 1.2 pA/pF, P = 0.5, cha>VAChT+(SYN) vs. control (cha/+ and UAS/+)). To verify that this experiment was sensitive enough to detect a change in response to ACh, I also carried out recordings in larvae which overexpressed the Da7 subunit of the nAChR in aCC motoneurons. This subunit is associated with excitability (Ping and Tsunoda, 2012) and so it was predicted that overexpressing it would cause an increase in the response to ACh. A significant increase in response to ACh was seen in these larvae (11.8 ± 0.8 vs. 8.8 ± 0.9 pA/pF, P = 0.02, RRa>nAChR_Da7 vs. control (RRa/+ and UAS/+), Fig. 3.9B). This suggests that changes in amplitude of minis and duration of SRCs caused by altering active VAChT level is a primarily presynaptic effect. It is noticeable in this experiment that amplitude, represented by current density, does not reach that of an SRC (Fig. 3.8F). This suggests that not all nAChR are activated and so calls into question the reliability of this experiment to measure the cells ability to respond to ACh.

Fig. 3.9

A  aCC response to ACh

B  Current density

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<tr>
<th>Condition</th>
<th>Current Density (pA/pF)</th>
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</tr>
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<td>RRa-GFP + 0.5µg CASPP</td>
<td>12.2 ± 1.7</td>
<td>6</td>
</tr>
<tr>
<td>cha&gt;VAChT+(SYN)</td>
<td>8.3 ± 1.2</td>
<td>13</td>
</tr>
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<td>cha&gt;VAChT+(SYN)</td>
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</tr>
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<td>13</td>
</tr>
<tr>
<td>RRa&gt;nAChR_Da7</td>
<td>12.2 ± 1.7</td>
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*  P < 0.05 vs. control
Figure 3.9  Manipulating the VACHT does not affect postsynaptic response to ACh

A. Response of aCC motoneurons in RRa-GFP larvae to different concentrations of ACh applied through perfusion. ACh at concentration 0.3mM was used for experiments (indicated by tick). B. CASPP-fed larvae do not show an altered sensitivity to ACh. Cha>VACHT^+(SYN) do not show altered sensitivity to ACh (control was cha/+ and UAS/+). RRa>nAChR_Da7 aCC motoneurons show an increased response to ACh (control was RRa/+ and UAS /+). Values are mean ± SEM.

3.2.8 Quantification of the overexpression of VACHT in cha>VACHT^+(SYN)

Quantification by qRT-PCR was carried out in order to evaluate the fold overexpression of VACHT achieved in cha>VACHT^+(SYN). Data was analysed using the Δ(ΔCt) method which calculates the difference in the number of thermal cycles required for fluorescence to be detected between gene of interest and control gene in test and control samples. This is then transformed using $2^{-\Delta(\Delta Ct)}$ to account for the assumption that PCR product doubles with every thermal cycle. Data is represented as fold change relative to a control expression of 1 and also after transforming data with log_2 to give a control value of 0, sometimes preferred for statistical analysis (Fig. 3.10). A significant up-regulation of transcript was found (fold difference: 9.69 ± 2.7, P = 0.002, log_2 difference: 3.1 ± 0.3, P < 0.001, n = 5). The VACHT and choline acetyltransferase (ChAT) genes share the same first exon and the remainder of the VACHT gene is within the first intron of ChAT (Kitamoto et al., 1998). For that reason I also carried out qRT-PCR for the ChAT transcript to ensure that expression of this gene was not inadvertently affected. There was no significant change in ChAT transcript (fold difference: 1.12 ± 0.16, log_2 difference: 0.10 ± 0.5, P = 1.0 for both, n = 5). Therefore, it can be concluded that increased mini frequency and amplitude is due to an overexpression of VACHT with no change to ChAT transcript level.
3.2.9 Expression of nAChR subunits in the aCC motoneuron

To better understand the cholinergic synapse between aCC and cholinergic interneurons, I investigated the subunit composition of the nAChRs expressed by aCC. The nAChR is a pentameric channel made up of 5 subunits, out of the possible 10, which are found in Drosophila (Dα1-7, Dβ1-3). This channel mediates the postsynaptic response to ACh and different subunits have been linked to specific characteristics of the ACh response. FACS was used to collect GFP-labelled aCC motoneurons from RRa-GFP third-instar larval CNSs, where only aCC is labelled by late third-instar. The number of cells collected ranged from 900-4500 in each sample (~20 per CNS). cDNA was synthesised from RNA extracted from these cells and also from RNA extracted from whole CNS. This was used to compare the relative abundance of nAChR subunits present in aCC cells compared to CNS.

The FACS-sorted aCC cell samples were, however, first tested for ‘purity’ by running PCR with primers specific for transcripts that should not be expressed in aCC cells: transcription factors Lim3 (Thor et al., 1999) and Islet (Thor and Thomas,
1997) and dopa decarboxylase (Ddc) (Beall and Hirsh, 1987), and for those that are present: Even-skipped (Eve) (Landgraf et al., 1999) and the vesicular glutamate transporter (VGLUT) (Mahr and Aberle, 2006). The house-keeping gene rp49 was also amplified for normalising. When the abundance of RNA present in aCC was calculated as a percentage of that found in the CNS, it was found that Lim3, Islet and Ddc were present in aCC cells at ≤ 12% of whole CNS, whereas Eve and VGLUT were enriched at 70% and 32% respectively (Fig. 3.11). This demonstrates only low levels of contamination by other neurons in the FACS-sorted aCC cells.

![Electrophoresis gel showing expression of control genes](image1.png)

![Presence in aCC cells](image2.png)

**Figure 3.11** Control genes in FACS sorted cells

**A.** Electrophoresis gel showing expression of Lim3, Islet, Eve, VGLUT, Ddc and rp49 in whole CNS and FACS sorted aCC cells. **B.** Percentage expression in aCC cells compared to whole CNS.

To determine the most abundant nAChR subunits expressed in aCC cells, PCR was run with primers specific for each of the subunits Da1-7, Dβ1-3. As all the subunits apart from Dβ3 appeared to be present at some level, possibly due to low levels of contamination, the house-keeping gene rp49 was also amplified and used to normalise. Three separately collected samples of FACS-sorted aCC cells were used and an average abundance of each subunit was calculated after normalising to
rp49. Using this method the most abundant subunits in aCC cells were Dα2, Dα5 and Dα6 (Fig. 3.12). This hierarchy is shown to be different to that found in whole CNS (Table 3.2), showing it is not the primer efficiencies responsible for this result. This suggests that Dα2, Dα5 and Dα6 may combine to make a pentameric receptor, or that they make up the majority of the nAChRs in combination with the other subunits expressed at lower levels.

**Figure 3.12** Expression of nAChR subunits in aCC cells

**A.** Electrophoresis gel showing expression of the 10 nAChR subunit encoding genes in whole CNS and **B.** FACS sorted aCC cells. Expression hierarchy indicates most abundant (1) to least abundant (10) subunit.
### 3.3 Discussion

#### 3.3.1 Blocking the VACHT causes mortality by reducing probability of SV release

The target protein of CASPP is confirmed as VACHT in mortality assays and blocking VACHT is shown to cause a sub-lethal effect of decreased speed and distance travelled in larval tracking. This is unsurprising given that *Drosophila* larval motoneurons receive cholinergic input (Baines *et al.*, 1999) and larvae in which cholinergic transmission is blocked are unable to crawl (Kitamoto, 2001). Therefore, this insecticide likely leads to mortality due to eventual paralysis of the larvae.

CASPP-fed larvae show a decrease in frequency but not amplitude of miniature synaptic events (minis). This is in agreement with studies at *Drosophila* and snake NMJs, where decreased VGLUT (*Drosophila*) and VACHT (snake) results in decreased frequency but not amplitude of miniature excitatory junctional potentials (Parsons *et al.*, 1999; Daniels *et al.*, 2006). However studies at the mouse and frog

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**Table 3.2** Hierarchy of expression of nAChR subunits in whole CNS and FACs sorted aCC cells of RRa-GFP larvae. Values in brackets represent expression relative to rp49.

<table>
<thead>
<tr>
<th>Hierarchy in CNS</th>
<th>Hierarchy in aCC cells</th>
</tr>
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<tbody>
<tr>
<td>Da5 (0.93 ± 0.036)</td>
<td>Da2 (0.94 ± 0.057)</td>
</tr>
<tr>
<td>Da1 (0.73 ± 0.146)</td>
<td>Da5 (0.88 ± 0.097)</td>
</tr>
<tr>
<td>Dβ1 (0.65 ± 0.196)</td>
<td>Da6 (0.76 ± 0.129)</td>
</tr>
<tr>
<td>Da7 (0.62 ± 0.206)</td>
<td>Da4 (0.39 ± 0.077)</td>
</tr>
<tr>
<td>Dβ2 (0.56 ± 0.116)</td>
<td>Da3 (0.35 ± 0.084)</td>
</tr>
<tr>
<td>Dβ3 (0.54 ± 0.142)</td>
<td>Dβ1 (0.29 ± 0.090)</td>
</tr>
<tr>
<td>Da2 (0.47 ± 0.091)</td>
<td>Da1 (0.27 ± 0.018)</td>
</tr>
<tr>
<td>Da4 (0.45 ± 0.091)</td>
<td>Da7 (0.25 ± 0.207)</td>
</tr>
<tr>
<td>Da6 (0.37 ± 0.085)</td>
<td>Dβ2 (0.21 ± 0.155)</td>
</tr>
<tr>
<td>Da3 (0.28 ± 0.072)</td>
<td>Dβ3 (0 ± 0)</td>
</tr>
</tbody>
</table>
NMJ and isolated vesicles from rat cerebral cortex and hippocampus also link decreased transporter with decreased neurotransmitter load (van der Kloot, 2000; Wilson et al., 2005; Prado et al., 2006; Lima et al., 2010 and Rodriguez et al., 2013). It may be that *Drosophila* SVs usually contain only one VAChT and so each SV is either loaded with ACh or empty if the VAChT is blocked by CASPP. This is believed to be the case at the *Drosophila* NMJ (Daniels et al., 2006). Conversely, the decrease in mini amplitude I have seen under vesamicol treatment would suggest blocking VAChT can affect amplitude of minis at *Drosophila* central synapses. However, the literature suggests another mode of action of vesamicol and it has been proposed to disrupt the proton gradient in SVs (Moriyama et al., 1991) and decrease the response of the NMJ to ACh in frog (Enomoto, 1988). Both of these mechanisms might lead to a decrease in mini amplitude. The decrease of SV release frequency is rescued in larvae which overexpress the wild-type VAChT in the presence of CASPP, validating this transporter as a target of this insecticidal compound. This also supports the hypothesis that decreased mini frequency in CASPP treated larvae is due to the effect of CASPP on blocking the VAChT.

Heterozygous VAChT mutants (VAChT+/−) do not show a change in mini frequency or amplitude. This may be expected because cholinergic synapse function has only been reported to fail in these flies under high frequency stimulation (200 Hz, 300 s) in the adult fly. Therefore, none of the methods intended to imitate CASPP was able to reliably reproduce the effect. However, the decrease in frequency seen in both CASPP and vesamicol treated larvae suggest that this is caused through a direct effect on VAChT.

Larvae which overexpress a resistant form of the VAChT not only show no decrease in mini amplitude when treated with CASPP but show an increased frequency of quantal release. Two possible explanations are: 1) The Y49N variant may interact with the endogenous wild-type VAChT, and in doing so its functionality is suppressed. Thus, when the wild-type VAChT is blocked by CASPP the Y49N function is released. 2) CASPP may still bind to VAChT<sup>Y49N</sup> but in doing so acts to increase the probability of SV release through promoting an interaction between VAChT and proteins involved in SV exocytosis or affect SV mobility to the membrane. An interaction between VAChT and synaptobrevin has been demonstrated in *C. elegans* (Sandoval et al., 2012) and may provide a possible mechanism. To fully understand the mechanism of resistance and any fitness cost associated with this mutation in the absence of CASPP this variant would need to be expressed without endogenous wild-type VAChT.
3.3.2 Blocking VACHT results in decreased frequency of SRCs

When VACHT is blocked by CASPP, action potential dependent spontaneous rhythmic currents (SRCs) become very infrequent or are not present and show a trend towards decreased amplitude. However, when the dose of CASPP is reduced by a small amount, SRCs are not different to control. At Drosophila and mouse NMJ, a reduction in endplate potential (EPP) amplitude is reported when VGLUT and VACHT are reduced, suggesting decreased quantal content (Daniels et al., 2006; Lima et al., 2010). As there is a significant reduction in mini frequency in CASPP larvae, but only a small reduction in amplitude of SRCs, it might suggest that the number of SVs released per SRC is increased to compensate for those that are empty. This result does not show the dose response expected from mortality assays. This may be caused by a difference in effect between chronic exposure (mortality assays) and acute exposure to CASPP. The lack of change in response to applied ACh supports increased SV release as opposed to an enhanced postsynaptic response. The reduction in SRC frequency may suggest a second action of CASPP, which has yet to be identified.

From the outset, it was predicted that VACHT overexpression would not change SRC amplitude due to homeostatic decrease in quantal content as has been reported at the Drosophila NMJ (Daniels et al., 2004). Although not statistically significant, this study reports a trend to increased SRC amplitude and a very significant increase in duration, suggesting that 1) quantal content remains the same and the increase is due to the observed increased amplitude of minis (Fig. 3.6A) or 2) that quantal content is increased due to increased probability of release; this will be further explored in chapter 4. The increased duration of SRCs is consistent with a change in postsynaptic response to ACh, however no change in response to applied ACh was observed. It is possible that SVs are released over a longer time period, leading to a prolonged depolarisation of the postsynaptic cell. This is supported by a reduction in duration after CASPP treatment. Dogma suggests that only a change to the nAChR can alter the time course of depolarisation of a cell. My results suggest a second mechanism through an increased duration of SV release (or decreased synchrony of firing of converging presynaptic inputs).

The increase in the inter-event duration between successive SRCs after an event of high amplitude suggests a possible negative feedback mechanism. Speculatively,
this may act via presynaptic muscarinic AChR (mAChR), which have been reported to play a role in modulating synaptic plasticity (Shinoe et al., 2005) and are present in presynaptic motoneurons at the mouse NMJ (Tomàs et al., 2014). The presence of a feedback mechanism may also facilitate changes in synaptic release in response to changes in ACh availability, for example due to insecticide application.

3.3.3 nAChR subunits Dα2, Dα5 and Dα6 are the most prevalent subunits in aCC

aCC motoneurons were shown to contain 9 out of 10 nAChR subunits present in the Drosophila genome. This result may be because these cells express all 9 subunits, albeit to differing levels. Alternatively, this result could be due to a low level of contamination in FACS-sorted cells. The most abundant subunits were calculated as those with the brightest bands in the gel when normalised to rp49 and were shown to be Dα2, Dα5 and Dα6. The Drosophila subunits Dα5, Dα6 and Dα7 are the most homologous to the vertebrate α7 subunit. This subunit is able to form a homomeric nAChR (Jones and Sattelle, 2010) as shown in Xenopus oocytes where Dα5 could be expressed as a homomeric channel or in combination with Dα6 (Lansdell et al., 2012). Therefore, it could be that Dα5 and Dα6 are able to form a functional channel in aCC, either by themselves or in a complex. The Dα2 subunit has been hypothesised to form channels with Dβ2 (Jepson et al., 2006) and Dα1 (Lansdell and Millar, 2000; Chamaon et al., 2002). In the hierarchy calculated for aCC cells, these subunits were in the bottom 4: this does not rule out their presence in a channel with Dα2 in the aCC cells. A study on nAChR subunit abundance in cha-1 positive (i.e. cholinergic) Drosophila cells found that all but Dα1 were present in the embryo, while in the larvae only Dα4, Dβ3 and one isoform of Dα6 were not present (Jepson, 2005). This also suggests that cha-1 neurons, similar to aCC, express a high number of nAChR subunits. Increased knowledge of subunit composition improves Drosophila as a model for insecticide mode of action studies because some insecticides which target the nAChRs, such as spinosad (Perry et al., 2007), are subunit specific. Therefore, this model could be used to narrow down specificity of nAChR-targeted insecticides with unknown mode of action.
3.4 Conclusions

This chapter demonstrates that the insecticidal compound CASPP, by targeting the VACHT, causes a decrease in quantal release of ACh but does not affect quantal size. These effects can be rescued by overexpression of the wild-type or resistant transporter. The resistant form of the transporter is not only immune to the effects of CASPP but also shows altered quantal release. Therefore *Drosophila* larvae proved to be a successful model in confirming mode of action of this compound. This chapter also reveals the predominant subunits comprising the nAChR in aCC motoneurons as Dα2, Dα5 and Dα6.
Chapter 4

The Effect of Overexpressing VACHT on Synaptic Vesicle-Mediated Release

4.1 Introduction

The VACHT has been shown, by previous studies and the preceding chapter, to play a significant role in modulating ACh release, in addition to SV loading. However, the link between VACHT and quantal release frequency (release of single loaded SVs) has not been fully explored.

Previous studies have investigated the effect of increased vesicular transporters in other systems. In *Xenopus* spinal neurons, increased VACHT leads to increased frequency and amplitude of miniature excitatory postsynaptic currents (Song *et al.*, 1997). When overexpressed in mouse, there is increased ACh release in the hippocampus (Nagy and Aubert, 2012). Similarly, overexpression of VGLUT in *Drosophila* motoneurons resulted in increased frequency and amplitude of miniature endplate junctional potentials at the NMJ (Daniels *et al.*, 2004). Cultured hippocampal neurons that overexpressed VGLUT showed increased amplitude but not frequency of miniature endplate potentials and an increase in glutamate load is also reported in isolated vesicles from rat cerebral cortex and hippocampus (Wilson *et al.*, 2005). With the exception of Nagy and Aubert (2012), the studies at cholinergic synapses are all carried out at peripheral synapses, namely the NMJ, and even the study of Nagy and Aubert does not explore quantal release of ACh at central synapses. Therefore, there is an almost total lack of studies exploring the effect of increased VACHT on quantal release of ACh at central cholinergic synapses. In this regard, *Drosophila* provides a good model with easily accessible and identified ACh receiving motoneurons within the larval CNS.

In *Drosophila* third-instar larvae, the majority of the CNS is thought to be cholinergic (Fig. 4.1, Gorczyca and Hall, 1987; Yasuyama and Salvettierra, 1999, Kim *et al.*, 2011). Immunostaining with anti-ChAT monoclonal antibody stains a large volume of third-instar larval brain, including the calyces, cortices associated with larval
central brain and the majority of the neuropil (Yasuyama and Salvaterra, 1999). In the ventral nerve cord, there is staining through almost the entire neuropil and labelled fibers run through the cortices to the neuropil region (Yasuyama and Salvaterra, 1999). Figure 4.1 shows cholinergic neurons, identified by cha-GAL4 driven expression of GFP and immunostaining against ChAT, overlap completely with synapses identified using antibodies against active zone markers bruchpilot and synapsin. ACh is the main excitatory neurotransmitter in the Drosophila CNS (Baines and Bate, 1998; Lee and O'Dowd, 1999). The cholinergic system plays a vital role in locomotion (Kitamoto, 2000; Baines and Pym, 2006; Landgraf and Thor, 2006) and significant roles in memory, copulation, neuronal plasticity and associative learning (Gu and O'Dowd, 2003; Acebes et al., 2004; Gu and O'Dowd, 2006, Gauthier, 2010; Dupuis et al., 2012).

This study has shown that increased VACHT in Drosophila produces an increase in ACh release at the synapse (chapter 3.2.4, Fig. 3.6). This current chapter aims to further explore the mechanism by which overexpressing VACHT causes increased ACh release. This is achieved through a combination of electrophysiology, pharmacology, electron microscopy and genetics. Understanding the mechanism by which VACHT influences neurotransmitter release will further knowledge of the role played by VACHT, for which there is gathering evidence is more than just SV loading.

**Figure 4.1** Presence of cholinergic neurons and synaptic markers in third-instar Drosophila CNS

A. Cholinergic neurons visualised by expressing a fluorescent membrane marker mCD8-GFP under control of a cholinergic specific GAL4 driver (cha). B. Cholinergic neurons visualised by immunostaining against ChAT. C,D. All synapses visualised by immunostaining against active zone markers bruchpilot (C) and synapsin (D). Scale bar is 200µm. Adapted from Kim et al., (2011).
4.2 Results

4.2.1 Mini amplitude, but not frequency, varies when VACHT is overexpressed

Overexpression of wild-type VACHT was achieved by crossing the cha\textsuperscript{B19}-GAL4 (cha) driver line to a UAS_VACHT\textsuperscript{+} line obtained from Syngenta (Sluder et al., 2012) and a UAS_VACHT\textsuperscript{+} line created at Manchester. Recordings from aCC using the UAS line from Syngenta were carried out 3 times over a 4 year period. To distinguish between different sets of recordings and the origin of the UAS line, they have been referred to as cha>VACHT\textsuperscript{+(SYN1)} recorded in 2012, cha>VACHT\textsuperscript{+(SYN2)} recorded in 2014 and cha>VACHT\textsuperscript{+(SYN3)} recorded in 2015, where SYN refers to Syngenta and the number references the different time points of recordings (note: these are all the same UAS-line). Recordings carried out using a UAS line created in the Baines group at Manchester are referred to as cha>VACHT\textsuperscript{+(M)}. This line was found necessary to produce for planned genetic crosses (to have the insert on the preferred chromosome). Sequencing of the UAS-VACHT\textsuperscript{+} from Syngenta found it to be a polymorphism that lacks one glutamine residue at position 549 (Fig. 4.2). The Manchester transgene was created using the CS sequence as a template and matched the database sequence (FlyBase ID FBpp0083086). It should be noted that because the Manchester UAS_VACHT\textsuperscript{+} was created some time into the project, the majority of experiments are carried out using UAS_VACHT\textsuperscript{+(SYN)} larvae.
### A

Sample VACHT nucleotide sequence

<table>
<thead>
<tr>
<th>VACHT</th>
<th>1623</th>
</tr>
</thead>
<tbody>
<tr>
<td>UAS_VACHT^{SYN}</td>
<td>1623</td>
</tr>
</tbody>
</table>

```
gccccgtgtggccaatcccttccagcagcagcagcagcaacaac
gccccgtgtggccaatcccttccagcagcagcagcagcaacaac
```

### B

VACHT amino acid sequence

| 1  | M A S F Q I P V I N L E V R E  V K D I V W E K I Q E P V N Q R R L I L V I V S I A L |
| 87 | H H D H H G Q D S A T G I L F A S K A I V Q L M V N P F S G G L I D K I G Y D L P |
| 128| M M I G L T I M F S T A V F A G G S S Y V L F A R S L Q G A S A F A D T A |
| 292| D N M T D N W K I G M V W L P A F F P H V L G V I T V K M A R Y P O H Q W |
| 332| L M A A G G A L A G F S C F I I P F C S G Y K M L M L P I C V I C F G I A L I D T A |
| 418| E A I G F T A L N F L I A F S N L A Y V P V L R K L R N I Y D F K P F E N E A N I L |
| 460| M Q D P P N K E Y Q T Y V M H D Q K P E G V K N H E L Y Q G Q Q Y Q Q E Q E |
| 499| T N L D D Q Y E Y Q Y Q Q Y Q Q Y Q D Q Y Q P Y Q E Q Q G S Y |
| 537| A P Q G Q P R V A N P F Q Q Q Q Q Q Q Q Q Q V Q S R G P A A P A N P F |
| 575| R Q G F |

**Figure 4.2** UAS_VACHT^{+SYN} lacks 3 base pairs which correspond to a missing glutamine (Q) amino acid in the translated sequence

**A.** Part of the VACHT nucleotide sequence showing 3 missing nucleotides in UAS_VACHT^{+SYN}. **B.** VACHT amino acid sequence. Red box indicates glutamine which is missing in UAS_VACHT^{+SYN}.

The absence of the single glutamine from the Syngenta VACHT transgene may be significant. At different time points of recording, the effect of overexpression of UAS_VACHT^{+SYN} was found to be variable: specifically, the effect on amplitude of spontaneous quantal release of neurotransmitter (minis) (Fig. 4.3). Initially, cha>VACHT^{+SYN} showed only a significant increase in frequency, but not amplitude of minis (amplitude: 8.28 ± 0.43 vs. 6.65 ± 0.63, P = 0.06, frequency: 56.13 ± 7.55 vs. 16.15 ± 2.7, P = 0.0005, cha>VACHT^{+SYN} vs. control, n = 9 for both. Control was RRa-GFP). Subsequent recordings showed an increase in
amplitude as well as frequency (amplitude: $15.29 \pm 1.74$ vs. $7.66 \pm 0.43$, $P = 0.006$, frequency: $98.67 \pm 17.25$ vs. $26.78 \pm 4.2$, $P = 0.008$, cha$>$VACHT$^{(S\text{YN}2)}$ vs. control, $n = 6$ and 12 respectively. Control was cha$/$ and UAS_VACHT$^+/+$). The most recent recordings, similarly show both an increase in amplitude and frequency (amplitude: $10.17 \pm 0.57$ vs. $7.36 \pm 0.36$, $P < 0.001$, frequency: $135.76 \pm 16.11$ vs. $63.5 \pm 8.90$, $P = 0.001$, cha$>$VACHT$^{(S\text{YN}3)}$ vs. control, $n = 10$ and 11 respectively. Control was cha$/$ and UAS_VACHT$^+/+$). Combining the data from all three sets of recordings shows a significant increase in amplitude and frequency (amplitude: $10.7 \pm 0.7$ vs. $7.3 \pm 0.3$, $P < 0.001$, frequency: $98.2 \pm 10.5$ vs. $36.4 \pm 5.0$, $P < 0.001$, cha$>$VACHT$^{(S\text{YN})}$ vs. control, $n = 25$ and 32 respectively). I therefore conclude that overexpressing the Syngenta variant of VACHT leads to increased amplitude as well as frequency of minis.

Recordings made from transgenic flies which were created by the Baines group in Manchester (denoted VACHT$^{(M)}$) consistently showed an increase in frequency but not amplitude of minis (amplitude: $8.22 \pm 0.31$ vs. $7.77 \pm 0.42$, $P = 0.3$, frequency: $100.5 \pm 6.86$ vs. $61.51 \pm 9.35$, $P = 0.003$, cha$>$VACHT$^{(M)}$ vs. control, $n = 11$ for both. Control was cha$/$ and UAS_VACHT$^+/+$). This result has been verified independently in the Baines Group (S Vernon, unpublished data). The difference between the Syngenta and Manchester versions of VACHT$^*$ appears to be independent of expression level (Fig. 4.3C) and VACHT in cha$>$VACHT$^{(M)}$ is up-regulated significantly when change in expression is transformed by log2 (log2 difference: $2.8 \pm 0.6$, $P = 0.01$). Up-regulation is not significant when fold change in expression is considered (fold difference: $8.3 \pm 2.6$, $P = 0.4$). However, this is likely due to the sample size of 3. This evidence that RNA expression is not different between the 2 transgenes does not rule out the possibility that there is a change in amount of functional protein produced.

Regardless of effect on mini amplitude, all averaged data sets, with both UAS_transgenes, show an increase in mini frequency. Therefore, I can confidently conclude that the postsynaptic motoneuron (in this instance aCC) is receiving increased exposure to synaptically-released ACh in cha$>$VACHT$^*$ larvae. I have previously shown in chapter 3 (section 3.6, Fig. 3.8) that the aCC motoneuron does not show a change in the amplitude of nAChR mediated depolarisation in response to applied ACh under these conditions. However, the extent to which this neuron is depolarised by ACh (e.g. how easily the threshold for action potential firing is reached) was not determined. This has been investigated by measuring the number of action potentials produced in response to injection of constant current.
A Amplitude

\[ \text{Amplitude (pA)} \]

\[ n = 6 \quad n = 10 \quad n = 9 \quad n = 11 \]

Combined

B Frequency

\[ \text{Frequency (min}^{-1} \)]

\[ n = 25 \quad n = 11 \]

C Relative fold change in expression

\[ 2^{\Delta(\Delta Ct)} \]

\[ n = 3 \quad n = 5 \quad n = 3 \]

Change in expression (log$_2$)
**Figure 4.3** Effect of overexpressing VACHT on mini amplitude and frequency

A. Amplitude of minis, recorded from postsynaptic aCC neurons, following expression of *cha>*VACHT<sup>+</sup>(SYN) and *cha>*VACHT<sup>+</sup>(M). B. Frequency of minis from the same recordings. The expression of *cha>*VACHT<sup>+</sup>(SYN) was repeated at 3 different time points and each individual data set (denoted SYN1-3) is shown. The year the experiment was performed is labelled within each column. The combined data from *cha>*VACHT<sup>+</sup>(SYN) is also shown (calculated by averaging data from all cells, labelled years 2012-2015). C. Expression of the VACHT transcript is similar between *cha>*VACHT<sup>+</sup>(SYN) and *cha>*VACHT<sup>+</sup>(M). Dotted lines represents control values, asterisk represents significance compared to control. Values are mean ± SEM.

### 4.2.2 Excitability of the postsynaptic motoneuron remains unchanged

To investigate if increased release of ACh, caused by overexpression of VACHT<sup>+</sup>, alters action potential firing of the affected postsynaptic motoneurons, the number of action potentials evoked by injected current in aCC was measured. Cells were recorded in current clamp mode and the membrane potential was maintained at -60 mV (by injection of small amounts of current). Current steps were injected to elicit action potential firing from this maintained baseline (-4 to +64 pA/500 ms, Δ4 pA). The number of action potentials fired at each step was counted (Fig. 4.4). It was found that overexpressing VACHT<sup>+</sup>(SYN) did not change the excitability of the postsynaptic aCC motoneuron. There was no significant difference between number of action potentials evoked at any current injected between *cha>*VACHT<sup>+</sup>(SYN) and control (P > 0.05 in all cases, Fig. 4.4C, control was cha/+ and UAS_VACHT<sup>+</sup>(SYN)/+).

The resting membrane potential of each cell was also measured before being adjusted to -60 mV (Fig. 4.4D), no significant difference was found (-48.5 ± 1.38 vs. -52.0 ± 1.16 mV, *cha>*VACHT<sup>+</sup>(SYN) vs. control, P > 0.05). This suggests that increased exposure of the post-synaptic motoneuron to released cholinergic SVs has not altered membrane excitability as measured by the firing of action potentials. VACHT<sup>+</sup>(M) has not been tested.
Figure 4.4 Membrane excitability is not changed following overexpression of VACHT+(SYN). aCC motoneuron response to injected current in 4 pA steps and resting membrane potential in cha>VACHT+(SYN) larvae

A. Current step protocol used to depolarise recorded cells. B. Example trace showing action potentials produced at 4 current steps. Red arrows indicate current injected. C. The number of action potentials produced at each current step was not different between control and cha>VACHT+(SYN) larvae. D. Resting membrane potential of cells on breakthrough (i.e. before current clamp was applied to establish a reference potential of -60 mV) was not different between control and cha>VACHT+(SYN). Values are mean ± SEM
4.2.3 Investigating vesicle number

My previous results show that overexpressing VACHT*\(^{(SYN)}\) consistently causes an increase in frequency of minis, whilst the spiroindoline compound 5Cl-CASPP (CASPP) causes a decrease in mini frequency. To determine if there is a change in the number of loaded vesicles (quanta), a number of methods were tried in order to measure SV number. This included using electron microscopy to estimate number of SVs per active zone and the toxin bafilomycin A1 to prevent acidification of SVs and so prevent re-loading. A conditional shibire mutant to block endocytosis and so block recycling of SVs was used and, finally, channelrhodopsin (ChR2) to stimulate the release of SVs. The result of each of these approaches is described below.

4.2.3.1 Electron microscopy to determine effects to SV number

Electron microscopy was used to investigate SV number at the synapse. This also allowed me to study any effect on synapse morphology of cha>VACHT\(^*\) and CASPP-fed larvae. Third-instar larval CNSs were fixed as described in chapter 2.7. Control (cha), cha>VACHT\(^{(SYN)}\) and control acutely fed 0.5 µg CASPP were examined. Sections were taken from 3 CNSs per sample, from the approximate area in which electrophysiological recordings are made (dorsal neuropil, segments Th2-3, A1-3). The initial fixation protocol used only one exposure to osmium tetroxide and did not produce high clarity of images (Fig. 4.5A). A second protocol, which incubated with osmium tetroxide (OsO\(_4\)), thiocarbohydrazide and a second incubation with OsO\(_4\) greatly increased membrane contrast and produced images with clearly visible SVs (Fig. 4.5B).
Figure 4.5 Two fixation protocols gave different clarity of images

A. Protocol 1 did not provide optimal clarity of SVs. B. Protocol 2 gave high clarity of vesicles. Scale bar is 200 nm. Synaptic vesicles (SV) are indicated by thick black arrows, and dense core vesicles (DCV) by thick white arrows. Pre- and post-synaptic membranes (Pre-SM/Post-SM) are indicated by thin black and white arrows. Mitochondria (M) are also labelled.

Example micrographs with synapses and SVs labelled from each sample are shown in figures 4.6 to 4.8. Synapses or active zones (these terms will be used interchangeably) were identified by pre- and postsynaptic membranes with increased density of the membrane and clustering of SVs. In many cases a T-bar structure and synaptic cleft can also be clearly identified (Fig. 4.9A). Other visible structures include mitochondria, dense core vesicles (DCVs) identified by their darker colour, and microtubules. In sections where microtubules were cut across the width of the tube, they can be distinguished from SVs due to their small size (~20nm). Figure 4.9A shows the range of synapse structures observed, including
different T-bar structures. The T-bar is thought to be involved in vesicle docking (Wichmann et al., 2001).

Fig. 4.6 Control micrographs
Figure 4.6  Micrographs showing synapses in control CNS

Example micrographs taken at 11,000X magnification in cha larvae, Scale bar is 200 nm in all images. * indicates synapses/active zones and arrows indicate vesicles.

Fig. 4.7  
Control + 0.5µg CASPP micrographs
Figure 4.7  Micrographs showing synapses in CASPP treated CNS

Example micrographs taken at 11,000X magnification of CNS of control larvae acutely fed 0.5 µg CASPP. Scale bar is 200 nm in all images. * indicates synapses/active zones and arrows indicate vesicles.

Fig. 4.8  Cha–VACHT+(SYN) micrographs
Figure 4.8  Micrographs showing synapses in cha>VACHT\textsuperscript{(SYN)}

Example micrographs taken at 11,000X magnification of cha>VACHT\textsuperscript{(SYN)} larval CNS. Scale bar is 200 nm in all images. * indicates synapses/active zones and arrows indicate vesicles.

Images were analyzed using Image J. The length of the active zone was measured and all SVs within 200 nm of the synaptic membrane were included in the analysis. This was to avoid including SVs that may be closer to a neighbouring synapse. Figure 4.9B illustrates how synapses were analyzed. A line function was used to draw the length of the membrane at the synapse and all nearby SVs were circled. The line function was then used to draw a line from the centre of each vesicle to the closest point of the membrane and only SVs within 200 nm were included in the analysis (Fig. 4.9B). Measurement of SV size, distribution with respect to the active zone and active zone length did not differ between control, cha>VACHT\textsuperscript{(SYN)} and control larvae fed CASPP (P > 0.5, Fig. 4.11, Table 4.1). This suggests that altered release of ACh is not due to changes in vesicle volume or an altered number of SVs at the active zone. The prevalence of a T-bar structure at each active zone was also recorded, it was found that T-bars were present at 45.5% of active zones in control larvae, 56.7% in CASPP-fed larvae and 48.8% in cha>VACHT\textsuperscript{(SYN)}.

To assess if the density of active zones differed between groups, random images were taken at a lower magnification (Fig. 4.10). There was no change in active zone density (p > 0.1, Fig. 4.11E, Table 4.1). This indicates that the change in frequency seen in minis is likely not due to a change in the number of active zones.

Table 4.1  Summary of electron microscopy analysis. Values describe results of analysis of vesicles and active zones morphology and density in cha (control), cha fed CASPP and in larvae which overexpress wild-type VACHT. Values are mean ± SEM.

<table>
<thead>
<tr>
<th></th>
<th>cha</th>
<th>cha + 0.5 µg CASPP</th>
<th>cha&gt;VACHT\textsuperscript{(SYN)}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>19.0 ± 0.9</td>
<td>18.5 ± 0.9</td>
<td>18.2 ± 0.8</td>
</tr>
<tr>
<td>Diameter (nm)</td>
<td>36.8 ± 0.7</td>
<td>38.5 ± 0.5</td>
<td>38.5 ± 0.6</td>
</tr>
<tr>
<td>Distance from membrane (nm)</td>
<td>112.9 ± 2.8</td>
<td>116.4 ± 2.2</td>
<td>112.2 ± 2.4</td>
</tr>
<tr>
<td>Length of membrane (nm)</td>
<td>256.5 ± 20.7</td>
<td>268.2 ± 13.9</td>
<td>266.0 ± 12.3</td>
</tr>
<tr>
<td>Number active zones/10µM&lt;sup&gt;+&lt;/sup&gt;</td>
<td>2.1 ± 0.1</td>
<td>2.1 ± 0.1</td>
<td>2.5 ± 0.1</td>
</tr>
</tbody>
</table>
Figure 4.9 Micrographs illustrating range of synapse structures observed and analysis of synapse morphology

A. Micrographs illustrating variation of synapse/active zones structure. Scale bar is 200 nm. Synaptic vesicles (SV) are indicated by thick black arrows, and T-bar structures by thick white arrows. Pre- and post- synaptic membranes (Pre-SM/Post-SM) are indicated by thin black and white arrows. B. Examples of how synapse morphology was analysed. Images are in pairs with unanalysed images on the left and the same analysed images on the right. Scale bar is 200 nm.
Figure 4.10  Example micrographs and analysis of active zone density

A - C. Micrographs taken at 1900X magnification of control, control acutely fed 0.5 \( \mu \text{g} \) CASPP and \( \text{cha}> \text{VACHT}^{(\text{SYN})} \) CNS. Three active zones per image are indicated by white arrows.
Fig. 4.11

A. Distance from membrane

B. Length of active zone

C. Number of vesicles per active zone

D. Vesicle diameter

E. Density of active zones
**Figure 4.11** Altering VACHT does not affect synapse structure

**A.** There was no significant difference in the mean distance of SVs from the membrane of the active zone between the three groups. **B.** There was no significant difference in length of the active zone between the three groups. **C.** There was no significant difference in the number of SVs per active zone between the three groups. **D.** There was no significant difference in SV diameter between the three groups. **E.** There was no significant difference in active zone density between the three groups. Control was *cha*. Values are mean ± SEM

### 4.2.3.2 Number of loaded SVs remains unchanged when VACHT is overexpressed

Electron microscopy indicates that there is no change in vesicle size or number when levels of VACHT are manipulated. To further explore if there is a change in quanta (i.e. number of ACh-containing releasable vesicles) I recorded action potential dependent synaptic currents (SRCs) from aCC in the presence of bafilomycin A1. Bafilomycin prevents the reloading of vesicles by blocking the proton pump and so preventing transport of ACh into the vesicle (Bowman *et al*., 1988). SRC amplitudes can be seen to continually run down and are absent following ~20 min exposure to bafilomycin (Fig. 4.12). Recordings were thus analysed for 20 minutes from application of bafilomycin. To estimate the number of quanta (i.e. SVs) released during this time, the area of all SRCs recorded was measured and summed and then divided by the average area of mini’s recorded under exposure to bafilomycin (in the presence of TTX). Area was used instead of amplitude as *cha>*VACHT*(SYN)* larvae show events which are longer in duration with no change in amplitude (chapter 3, Fig. 3.8). Total area of SRC’s in *cha>*VACHT*(SYN)* larvae was increased compared to control (2.38 x 10^7 ± 0.4 x 10^7 vs. 1.0 x 10^7 ± 0.1 x 10^7 pA/ms, *P* = 0.01, *cha>*VACHT*(SYN)* vs. control, Fig. 4.13A, control was *cha/+* and UAS VACHT*(SYN)/+). Mean mini area was also markedly increased in *cha>*VACHT*(SYN)* larvae (141.0 ± 11.8 vs. 52.9 ± 6.2 pA/ms, *P* = 0.00001, *cha>*VACHT*(SYN)* vs. control, Fig. 4.13B) indicative that each SV released more ACh. Calculation of released quanta, during the 20 min bafilomycin exposure, shows that the number of SVs released (i.e. ACh filled vesicles) was not significantly different between *cha>*VACHT*(SYN)* and control (1.9 x 10^5 ± 0.3 x 10^5 vs. 2.0 x 10^5 ± 0.2 x 10^5 total vesicles released, *P* = 0.8, *cha>*VACHT*(SYN)* vs. control, Fig. 4.13C). Number of quanta per SRC, calculated by dividing total number of...
quanta by SRC frequency, was also not significantly different in cha\textgreater VACHT\textsuperscript{(SYN)} vs. control (584.8 ± 116.1 vs. 707.3 ± 117.0 quanta per SRC, P = 0.5, Fig. 4.13D). These results are consistent with increased levels of ACh per vesicle following overexpression of VACHT\textsuperscript{(SYN)} which causes increased mini amplitude but no change to total number of loaded SVs (supported by electron microscopy which showed no change to SV number).

By contrast, SRC total area was decreased in CASPP-fed larvae (0.2 x 10\textsuperscript{7} ± 0.09 x 10\textsuperscript{7} vs. 1.3 x 10\textsuperscript{7} ± 0.3 x 10\textsuperscript{7} pA/ms, P = 0.008, 0.5 µg/ml CASPP vs. control, Fig. 4.19A, control was unfed RRa-GFP). Average area of minis recorded in the presence of CASPP showed no difference to controls (34.4 ± 3.1 vs. 50.4 ± 10.1 pA/ms, P = 0.2, 0.5 µg/ml CASPP vs. control, Fig. 4.13B). Dividing total SRC area by averaged mini area for this condition shows that the number of quanta released during bafilomycin exposure was significantly reduced (0.5 x 10\textsuperscript{5} ± 0.2 x 10\textsuperscript{5} vs 2.5 x 10\textsuperscript{5} ± 0.6 x 10\textsuperscript{5} total vesicles released, P = 0.01, 0.5 µg CASPP vs. control, Fig. 4.13C), supporting the model that there is a decrease in the number of quanta (i.e. filled vesicles) under these conditions. However, the number of quanta per SRC was not significantly decreased in CASPP-fed vs. control (674.27 ± 234.02 vs. 955.2 ± 208.5, quanta per SRC, P = 0.4, 0.5 µg CASPP vs. control, Fig. 4.13D).
Endogenous SRC recordings in presence of bafilomycin

Fig. 4.12
**Figure 4.12** Bafilomycin causes a rundown of SRCs

Endogenous SRCs recorded in an aCC motoneuron at time points 0 – 2 minutes, 8 – 10 minutes and 18 - 20 minutes. Traces illustrate rundown of events in control (vehicle) and in the presence of bafilomycin in control, control acutely fed CASPP and cha>VACHT^{(+SYN)} larvae. It should be noted that before addition of bafilomycin, 4 out of 6 recordings of CASPP-fed larvae showed no events above 100 pA. The appearance of larger events after adding bafilomycin is caused by the DMSO required to solubilise bafilomycin and was at 0.4% final concentration. DMSO was also included in all control recordings.

![Bar charts](image_url)

**Fig. 4.13**

A. Total SRC area

B. Mean mini area

C. Total number of quanta

D. Quanta per SRC

* N = 9, ** N = 6, *** N = 7, n.s.
Figure 4.13  Number of ACh containing vesicles is unchanged in cha-VACH'T+ larvae and decreased in CASPP treated larvae

A. Total area of all SRCs over the 20 minute recording is significantly reduced in CASPP fed larvae and significantly increased in cha-VACH'T+(SYN) larvae. B. Mean mini area is not significantly different in larvae acutely fed CASPP but is significantly increased in cha-VACH'T+(SYN) larvae. C. Number of quanta (ACh containing SVs) released over 20 minutes is significantly decreased in larvae acutely fed CASPP but is not different in cha-VACH'T+(SYN) larvae. D. Number of quanta per SRC is not significantly reduced in either CASPP-fed larvae or cha-VACH'T+(SYN). Bars are in pairs with CASPP control and test group on the left and cha-VACH'T+(SYN) control on the right. Control for cha-VACH'T+(SYN) is cha and UAS_VACH'T+(SYN) crossed with CS. Values are mean ± SEM.

It should be noted that the presence of SRCs, which are not normally present at this concentration of CASPP, is due to the presence of DMSO. DMSO causes an increase in size and/or duration of SRCS (Fig. 4.14). This has previously been reported in other systems and is believed to be caused by a combination of an inhibitory effect on AChE and a facilitating effect on either calcium entry or binding (Evans and Jaggard., 1973; Matsumoto et al., 1985; McLarnon et al., 1986). This effect may have been beneficial in this experiment reducing the time for available vesicles to be released.

Figure 4.14  DMSO causes an increase in amplitude and/or duration of SRCs

A. Addition of bafilomycin causes increased size of SRCs. B. Addition of DMSO vehicle also causes increased size of SRCs.

Taken together, my analysis suggests that manipulating SV loading with either VACH'T+ overexpression or CASPP does not alter the number of loaded SVs.
(quanta) released per SRC in either condition. Moreover, whilst analysis of mini area clearly shows that VACHT+(SYN) overexpression results in increased filling of SVs, lack of change to this parameter in CASPP-fed larvae suggests that block of VACHT leads to unfilled vesicles. Both outcomes are consistent with this transporter representing a rate-limiting step in the vesicle release cycle (Prado et al., 2013).

4.2.3.3 Shibire\textsuperscript{ts} and channelrhodopsin

The third and fourth methods used to assess quantal number used a temperature sensitive inhibitor of SV endocytosis and channelrhodopsin, respectively. Neither was successful due to unwanted side effects of experimental procedure. However, because some interesting results were found, these experiments are described below.

The \textit{shibire}\textsuperscript{ts} (\textit{shi}\textsuperscript{ts2}) fly carries a mutation in the \textit{shibire} gene which encodes dynamin. Dynamin plays a role in clathrin-mediated endocytosis (De Camilli et al., 1995). At the restrictive temperature of 27°C (Krishnan et al., 2001) endocytosis of SVs is inhibited which quickly depletes presynaptic terminals of SVs. It was predicted that, while recording endogenous (action potential-dependent) spontaneous rhythmic currents (SRCs) in aCC motoneurons in larval CNS, if the temperature was increased to >27°C, recycling of SVs would be blocked and total SRC events after this point would represent available SVs in the readily-releasable pool. This would allow an estimate of number of releasable SVs to be determined (i.e. total SRC area / single quantal area).

After achieving a whole-cell patch and endogenous SRCs observed, the preparation was perfused with heated saline until the bath temperature reached 28-30°C. As can be seen in Figure 4.15A, SRC events were lost above 27°C and resumed once the temperature of the bath returned to <27°C. This was the predicted result, but nevertheless, to confirm that this was an effect of the \textit{shibire} mutant and not temperature, I repeated the recordings in wild-type (CS and OrR) and RRa-GFP larvae. In all cases SRCs were greatly reduced in size and frequency at temperatures above 26°C (Fig. 4.15B-D). This is a puzzling result which suggests that synaptic transmission in \textit{Drosophila} larval CNS is sensitive to temperature and, moreover, is reduced to almost nothing at temperatures above 26°C (this is particularly unexpected because \textit{Drosophila} live in tropical climates). This approach using \textit{shibire} was abandoned due to this unwanted effect of temperature.
Fig. 4.15

A. Shibire mutant (shits2) aCC

Temperature

Endogenous SRCs

B. Canton-S (wildtype) aCC

Temperature

Endogenous SRCs

C. Oregon-R (wildtype) aCC

Temperature

Endogenous SRCs

D. RRa-GFP aCC

Temperature

Endogenous SRCs
Figure 4.15  SRC recordings are sensitive to external temperature. Recordings from aCC show an effect of changing temperature of the external saline

A. Shibirets mutants show a loss of SRCs at 26 – 28°C which return once the temperature drops back below 26°C.  B. CS wild-type larvae also show loss of large SRCs at >26°C which in this case do not return to full size upon cooling.  C. OrR wild-type show a reduction in size and frequency of SRCs at 26 – 28°C which return to normal below 26°C.  D. RRa-GFP larvae also show a reduction in size and frequency of SRCs at 26–28°C which return to normal on cooling.

My second approach, to evoke greater exocytosis of vesicles at increased temperature (in the hope of combining this technique with shibire), I used channelrhodopsin (ChR2H134R). Channelrhodopsin is a light sensitive cationic channel originally found in the green algae, Chlamydomonas reinhardtii (Pulver et al., 2009). The channel opens in response to blue light (~ 450nm) and causes depolarisation and action potential firing in neurons (Nagel et al., 2003; Pulver et al., 2009; Lin., 2010). ChR2H134R was expressed in cholinergic neurons using cha. SRC-like events can be produced in the aCC motoneuron in cha>ChR2H134R larvae by applying short pulses of blue light while in whole-cell voltage clamp (Fig. 4.16A-B). Pulses (2 ms) were applied at 1 Hz; above this frequency induced events did not follow 1:1 with light pulses (representing time taken to reconstitute the all-trans retinal cofactor). To evoke SV release, the saline surrounding the CNS was first heated to 28°C (where no endogenous SRCs occur) before a stimulus train of light pulses was applied (Fig. 4.16C-D). It can be seen that, at elevated temperature, although each light pulse elicits a response the induced events are small and short in duration. At both room temperature and 28°C the events provoked by the stimulus train show a larger first event in comparison to event 2 (Fig. 4.16B,D). However, the difference in amplitude between events 1 and 2 between the two temperatures was not significant (184.0 ± 51.2 vs. 259.3 ± 75.1 pA, 28°C vs. room temperature, P = 0.4, Fig. 4.17A). This suggests that this effect is not a result of temperature. However, there was a significant decrease in the duration of induced SRCs at 28°C vs. room temperature (Fig. 4.17B, taken from a sample of 15 events, 2.5 minutes into the recording, 0.38 ± 0.03 vs. 0.76 ± 0.04 sec, 28°C vs. room temperature, P < 0.001). Because of the difference in SRCs at elevated temperatures, I was not confident that this represented a good way to continue and, thus, I also abandoned this approach.
Fig. 4.16

A

Temperature
Stimulated SRGs

Cha>ChR2H134R, room temperature, aCC
20 - 22°C

200 pA
10 sec

B

Temperature
Stimulated SRGs

Cha>ChR2H134R, room temperature, aCC
20 - 22°C

200 pA
1 sec

Event 1
Event 2

Stimulus train
(1 Hz, 2ms duration)

C

Temperature
Stimulated SRGs

Cha>ChR2H134R, heated, aCC
28°C

200 pA
10 sec

D

Temperature
Stimulated SRGs

Cha>ChR2H134R, heated, aCC
28°C

200 pA
2 sec

Event 1
Event 2

Stimulus train
(1 Hz, 2ms duration)
**Figure 4.16** Channelrhodopsin can be used to evoke presynaptic cholinergic release

Stimulated SRCs in aCC at room temperature (20 - 22°C) and at 28°C in cha>ChR2<sup>H134R</sup> larvae in response to blue light pulses (2 ms). **A.** Shows an ~2 min recording of SRCs, evoked by blue light pulses at 1 Hz at room temperature. **B.** Expanded time base to show shape of stimulated SRCs and demonstrates stimulus train as well as illustrating the drop in amplitude between events 1 and 2 at room temperature. **C.** shows SRCs evoked by stimulated by blue light pulses at 1 Hz at 28°C. **D.** Expanded time base to show shape of stimulated SRCs and demonstrates stimulus train as well as illustrating the drop in amplitude between events 1 and 2 at 28°C.

---

**Figure 4.17** SRC duration is influenced by temperature in wild-type

Analysis of cha>ChR2<sup>H134R</sup> events in aCC at room temperature and when heated to 28°C. **A.** The drop in amplitude between events 1 and 2 was not significantly different when the preparation was at room temperature or 28°C. **B.** Duration was significantly decreased at 28°C. Values are mean ± SEM

To determine if cha>ChR2<sup>H134R</sup> alone could be used to assess SV number (by inducing SV release at a rate that exceeded recycling) records were made at room temperature when available SVs were restricted in larvae acutely fed 0.5 µg CASPP for 24 hours (Fig. 4.18A-B). If SV release exceeded recycling it was expected that a run-down of events would be observed. All ChR2-induced SRC events in each recording were normalised to the mean amplitude calculated from SRC events 2-6 (to avoid including the larger first event). CASPP-fed larvae appear to show an increased rundown of events over a 3 minute period and average amplitude is
decreased (0.65 ± 0.01 vs. 0.77 ± 0.01, P = 0.2 x 10^{-19}, 0.5 µg CASPP vs. control, n = 210 and 205 total events, Fig. 4.18C). Although number of events was high, the number of cells recorded from was very low so although suggestive of a difference, this test is not statistically reliable. The same experiment was carried out at 28°C to determine if a rundown and eventual loss of events could be seen. However, evoked SRC events were recorded at a steady amplitude for >10 minutes (Fig. 4.19A), albeit with reduced amplitude and duration due to elevated temperature. This suggests that ChR2 cannot induce enough vesicle release to cause rundown of events within a reasonable period of time.

Fig. 4.18

A

*Cha>ChR2*{H134R}, room temperature + 0.5µg CASPP, aCC

Temperature

| 20 - 22°C |

Stimulated SRCs

200 pA

10 sec

B

*Cha>ChR2*{H134R}, room temperature + 0.5µg CASPP, aCC

Temperature

| 20 - 22°C |

Stimulated SRCs

C

Normalised amplitude

- control, n = 2
- control + 0.5µg CASPP, n = 4
Figure 4.18  CASPP treatment is able to increase rundown in amplitude of events

Stimulated SRCs in aCC at room temperature (20 – 22°C) in cha-ChR2<sup>H134R</sup> larvae acutely fed 0.5 µg CASPP. A. Trace shows ~2 min recording of SRC events evoked by blue light pulses at 1 Hz. B. Expanded time base to show shape of events. C. Normalised amplitude of SRC-like events, in aCC, at room temperature from cha-ChR2<sup>H134R</sup> and cha-ChR2<sup>H134R</sup> acutely fed 10 µg/ml CASPP, stimulated by blue light pulses at 1 Hz. A bigger drop in amplitude can be seen in larvae fed CASPP and amplitudes are significantly different. Values are mean ± SEM

It is interesting to note that ChR2-induced SRCs in aCC in the cha-ChR2<sup>H134R</sup> background at 28°C are very similar in shape and size to similar evoked events in CASPP-fed larvae at room temperature (where control synaptic SRCs are normal, Fig. 4.19B-D). This was unexpected because this insecticide blocks most SRCs at this concentration (see chapter 3.2.6). Thus, to test that these events are caused by the release of ACh, I repeated the recordings in the presence of TTX, a sodium channel antagonist, and mecamylamine, a nAChR antagonist. Recordings were made in the aCC motoneuron as well as another motoneuron which can also be easily identified, the RP2 motoneuron. Surprisingly, light pulses (1 Hz) were able to induce SRC-like events in aCC in both TTX and mecamylamine treated CNS, but not in RP2 motoneurons (Fig. 4.20A-B). This suggests that the cha<sup>B19</sup>-GAL4 driver is also expressed (weakly) in the aCC motoneuron and the events recorded from this cell are caused by the direct expression/activation of ChR2 in this neuron. We have since confirmed this by expression of GFP. Hence, all recordings using aCC and ChR2 must be re-interpreted.
Fig. 4.19

A

Temperature

Simulated SRCs

28°C

B

C

D

Chₐ-ChR₂²₃⁴₅, heated + 0.5µg CASPP, aCC

Chₐ-ChR₂²₃⁴₅, heated

Chₐ-ChR₂²₃⁴₅, room temperature

Chₐ-ChR₂²₃⁴₅, room temperature

+ 0.5µg CASPP

100 pA

0.5 sec

50 pA

1 min

100 pA

0.5 sec
Figure 4.19  Events at 28°C are similar to events in CASPP treated larvae at room temperature (20-22°C)

A. A recording in an aCC motoneuron showing SRC-events in cha>ChR2^{H134R} fed 0.5 µg CASPP at 28°C remain constant for > 10 minutes. B. Cha>ChR2^{H134R} at room temperature (20 – 22°C). C. Cha>ChR2^{H134R} at 28°C. D. Cha>ChR2^{H134R} at room temperature, acutely fed 0.5 µg CASPP.
Fig. 4.20

A Room temperature + 2µM TTX

B Room temperature + 1mM mecamylamine

C Cha>ChR2^{H134R}, heated, RP2

D Cha>ChR2^{H134R}, heated + 1mM mecamylamine, RP2
Figure 4.20  aCC motoneurons in cha>ChR2\textsuperscript{H134R} respond to light pulses in the presence of synaptic signalling inhibitors

Stimulated SRC events, in the presence of TTX or mecamylamine, in aCC and RP2 motoneurons in a cha>ChR2\textsuperscript{H134R} background. A. Events in aCC (top) and RP2 (bottom) in the presence of 2 µM TTX. B. Events in aCC (top) and RP2 (bottom) in the presence of 1 mM mecamylamine. C. Stimulated SRC events in RP2 of cha>ChR2\textsuperscript{H134R} at 28°C, illustrating a drop in amplitude between events 1 and 2 and small size of events as seen in aCC motoneuron recordings. D. Stimulated SRCs in an RP2 motoneuron in cha>ChR2\textsuperscript{H134R} at 28°C with mecamylamine added during recording (red arrow), illustrates that stimulated events are caused by the release of ACh from presynaptic neurons.

Stimulated SRCs in RP2 motoneurons, which I conclude are due to presynaptically-released ACh, were recorded at 28°C. At this elevated temperature, the induced SRCs also showed a pronounced decrease in amplitude between first and second events and, by now characteristic, small amplitude (< 200 pA) and duration (Fig. 4.20C). Addition of mecamylamine during recording (Fig. 4.20D) confirms that these events are caused by release of ACh. However, these SRC-like events are, by comparison to wild-type, non-physiological. Time has not permitted a repeat of the above experiments using RP2, which does not express GAL4 in the cha\textsuperscript{B19}.GAL4. This, however, represents a possible way forward.

To conclude, attempts to use shibire and/or elevated temperature with, or without ChR2, were problematic and were not followed up.

4.2.4 Overexpressing VAChT causes an increased probability of release

Increased ACh load per SV is sufficient to explain increased amplitude of minis in cha>VAChT\textsuperscript{+}[SYN] larvae but does not account for the observed increase in mini frequency in both cha>VAChT\textsuperscript{+}[SYN] and cha>VAChT\textsuperscript{+}[M] larvae. Because electron microscopy data is consistent with no change in the number of SVs at the presynapse, VAChT seemingly acts to increase probability of SV release. This has been observed at peripheral cholinergic NMJs (Song et al., 1997; Daniels et al., 2004).
VAClT has been reported to bind to the SNARE protein synaptobrevin in *C. elegans* through a glycine residue at position 347 (Sandoval *et al.*, 2006). This interaction raises the possibility that VAClT and synaptobrevin interact at cholinergic synapses to link filling of vesicles to their release. My results showing altered frequency of minis are consistent with this hypothesis. The glycine at position 347 is well conserved and is present in *Drosophila* VAClT. Thus, a glycine (G) to arginine (R) substitution (VAClT$^{G342R}$, Fig. 4.21A) at this position was engineered, which Sandoval *et al* (2006) show is sufficient to prevent binding to synaptobrevin. Expression of VAClT$^{G342R(M)}$ in cholinergic neurons (*cha>*VAClT$^{G342R(M)}$) in *Drosophila* fails to increase the frequency of minis recorded in aCC. No effect on mini amplitude was also observed, which is consistent with the WT *cha>*VAClT$^{+/M}$ created at the same time (amplitude: 7.0 ± 0.3 vs. 6.7 ± 0.3 pA, P = 0.6; frequency: 69.5 ± 8.1 vs. 67.7 ± 7.5 per min, P = 0.9, control was *cha/+ UAS_VAClT$^{G342R/+}$, Fig. 4.21B,C). Absence of increased mini frequency is not due to the G342R variant not being expressed, shown by RT-qPCR (fold difference: 21.2 ± 4.4, P = 0.009, log$_2$ difference: 4.33 ± 0.34, P = 0.001, Fig. 4.21D-E).

However, I have yet to prove that a functional protein is produced.

These results suggest that in the *Drosophila* CNS, increased VAClT leads to increased release of quanta which is not caused by increased vesicle number, nor an increase in loaded vesicles. It proposes a secondary role for VAClT in promoting vesicle fusion.
Figure 4.21  VAChT\textsuperscript{G342R} does not increase mini frequency

A. Part of VAChT amino acid sequence indicating amino acid change G342R. B. Overexpressing VAChT\textsuperscript{G342R} does not significantly affect mini amplitude. C. Overexpressing VAChT\textsuperscript{G342R} does not significantly affect mini frequency. D-E. VAChT expression is greatly increased in cha>VAChT\textsuperscript{G342R(M)} larvae. Dotted line represents control expression of 1. Values are mean ± SEM.
4.3 Discussion

4.3.1 Overexpressing the VAChT causes an increase in mini frequency and inconsistently an increase in mini amplitude

The primary role of the VAChT is to load ACh into SVs (Usdin et al., 1995; Nguyen et al., 1998). This present study is the first to show the effect of overexpressing VAChT on ACh SV release at a central cholinergic synapse. Increased VAChT produced a reproducible increase in the frequency of action potential independent miniature synaptic currents (minis). Effect on mini amplitude was more variable and an effect only observed for a variant carrying a polymorphism of missing a glutamine residue. My results are in broad agreement with previous studies that, for example, show VAChT in cultured Xenopus spinal neurons is sufficient to increase quantal size and frequency of miniature excitatory postsynaptic currents (Song et al., 1997).

That the VAChT+ line provided by Syngenta, which has one fewer glutamine residues in a polyQ domain at the C-terminus, showed an overall increase in amplitude of minis is an interesting observation. By contrast, the VAChT+ line created in the Baines group, which has the full complement of 13 glutamine residues in the polyQ region does not affect mini amplitude when expressed. This line also produces a more modest increase in frequency. PCR-analysis rules out any effect on expression level. This poses the interesting possibility that the polyQ domain may play a role in VAChT function. Extended polyQ domains occur in many diseases such as Huntington’s where the polyQ domain has been linked with increased apoptosis in a yeast model (Sokolov et al., 2006). An extended polyQ domain is also implicated in spinocerebellar ataxia and may also inhibit gene expression (Lievens et al., 2005). However, very little is known about the function of normal length polyQ domains. PolyQ domains are found in proteins in mammals, invertebrates, plants and yeast (Schaefer et al., 2012) and are particularly common in Drosophila. Suggestions for the normal functioning of these polyQ domains include protein-protein interactions, transcriptional regulation, RNA binding and signalling. The number of glutamine residues may also be important for the exact function of the polyQ domain (Schaefer et al., 2012). The VAChT sequence in mouse, rat and human all contain a di-leucine motif within the cytoplasmic C-terminal which is important for localising VAChT to the SV membrane and also plays a role in endocytosis of SVs after neurotransmitter release through an
interaction with the AP-2 complex (Tan et al., 1998; Barbosa et al., 2002). This di-leucine motif is not present in Drosophila VACHT, whilst the polyQ region is unique to Drosophila, posing the possibility that this polyQ region may be responsible for VACHT localisation and SV endocytosis after fusion at the membrane. If the exact number of glutamine residues is important, it is possible that the Syngenta UAS_VACHT*, which lacks one glutamine, is more efficiently transported to the SV, resulting in increased VACHT per vesicle.

4.3.2 There is no change in excitability of the postsynaptic cell when VACHT is overexpressed

It has previously been reported in Drosophila and mammals that increased action potential dependent synaptic currents lead to a decrease in action potential firing in the postsynaptic cell through increasing the transcriptional regulator pumilio which suppresses sodium channel expression (Mee et al., 2004; Driscoll et al., 2013). Homeostasis can also occur by reduction of the resting membrane potential, as had been found at the Drosophila NMJ via activation of a calcium-dependent potassium channel (Gertner et al., 2014). This study shows that when mini frequency and SRC size (chapter 3.2.6) is increased there is no compensatory decrease in action potential firing or resting membrane potential in the postsynaptic aCC motoneuron. The increased frequency and amplitude of minis may not be enough to cause a postsynaptic decrease in excitability as these small currents do not cause enough depolarisation of the postsynaptic membrane to initiate action potentials. However, it was expected that increased SRC duration would cause a decrease in action potential firing and/or hyperpolarisation of resting membrane potential. It is possible that the relationship between amplitude of SRC and inter-event interval (increased amplitude leads to increased interval, chapter 3.2.8) is sufficient to maintain excitation within normal physiological boundaries. This suggests homeostatic regulation at this synapse is complex and not limited to regulation of ion channels or receptors.
4.3.3 Overexpressing VACHT does not increase SV number or volume

In this study, EM analysis suggests that when the VACHT is overexpressed, or blocked, there is no change to SV size or number. This is in agreement with a study at frog NMJ, where an increase in quantal size of > 3-fold, or a decrease by two-thirds, has no effect on vesicle size (Van der Kloot et al., 2002). However, at the Drosophila NMJ, SV diameter and number were reduced in a VGLUT mutant and diameter was increased when the VGLUT was overexpressed (Daniels et al., 2004, 2006). In VACHT knockdown mice, vesicle number remained the same but size decreased at the NMJ (Rodrigues et al., 2013). A conclusion of no increase in SV number is supported both by EM and by the finding that when vesicle recycling is blocked with bafilomycin, total number of vesicles released is unchanged when VACHT is overexpressed. My results are consistent with the possibility that SVs, at central cholinergic synapses, are not “full” and that increasing the ACh loaded into the vesicle (with VACHT^SYN) results in more ACh per SV rather than a change to vesicle volume. Blockade by CASPP, sufficient to reduce frequency but not mini amplitude, suggests that many of the vesicles in CASPP treated larvae are empty. There is a reduction in total vesicles released in CASPP-fed larvae when vesicle recycling is blocked, supporting the presence of empty vesicles. Significantly, it has been shown that empty vesicles are still able to undergo recycling (Parsons et al., 1999).

A drawback of my analysis is that individual synapses could not be unequivocally identified as cholinergic. However, because it is assumed that the majority of synapses in the CNS are cholinergic (Gorczyca and Hall, 1987; Yasuyama and Salvaterra, 1999), it was expected that any changes induced by altering the levels of active VACHT would be discernible. Nevertheless, I cannot discount the possibility that any alterations in cholinergic synapses were not detectable as they were diluted by the presence of non-cholinergic synapses.

This study endeavoured to use the conditional shibire mutant, which blocks synaptic transmission at restrictive temperatures due to prevention of vesicle endocytosis (Krishnan et al., 2001). Endogenous SRCs quickly ceased at the restrictive temperature in these mutants but were also greatly reduced in amplitude and frequency in control CNSs heated to the restrictive temperature. Another study which performed electrophysiology in Drosophila using the conditional shibire mutant at the restrictive temperature also reported quality issues in control
recordings. Even electrically evoked currents were found to rundown over time in control and were only of ~ 100 pA in size (Rohrbough and Broadie, 2002).

When ChR2 was used to drive SRC-like events at >28 °C, these events were of smaller size and amplitude than in preparations maintained at room temperature which suggests that synaptic release was still inhibited. Because Drosophila are able to be maintained at 29°C as larvae and adult flies, this suggests that the CNS is somehow protected from temperature extremes in the whole animal. In electrophysiological recordings the neurolemma surrounding the CNS is ruptured, which may lead to increased sensitivity of the CNS to temperature changes. Another limitation of ChR2 is that it has a high level of desensitisation (due to lack of availability of the all-trans retinal cofactor, Lin, 2011), which may explain the initial larger amplitude of the first event followed by a stable size of the following events and probably also accounts for a failure of 1:1 response to light pulse at frequencies above 1 Hz.

4.3.4 Overexpressing VACHT increases the probability of vesicle fusion

I have shown that increased spontaneous release frequency is not caused by an increase in the number of SVs at the active zone or active zone density. This poses the possibility that VACHT plays a second role in vesicle mobilisation or fusion. Other studies have previously hinted at such a second role: at the mouse NMJ there is altered distribution of vesicles in VACHT knock-down animals, but this was not recapitulated when VACHT was pharmacologically blocked (Rodrigues et al., 2013). In C. elegans a glycine to arginine amino acid substitution in VACHT prevents an interaction with synaptobrevin, a vesicle associated membrane protein that is pivotal for exocytosis (Link et al., 1992; Schiavo et al., 1992). This phenotype is rescued by a polarity changing isoleucine to aspartate substitution in synaptobrevin (Sandoval et al., 2006). The VACHT glycine residue is conserved in many species, including Drosophila, mouse, rat and human. That this study shows that VACHTG342R expression in Drosophila has no effect on vesicle release is consistent with this interaction and, further, adds in vivo experimental support for the hypothesis that this transporter regulates vesicle release through binding to synaptobrevin.
4.4 Conclusions

In conclusion, this chapter shows that VACChT can govern ACh release at a central cholinergic synapse in *Drosophila*. Increased VACChT causes increased quantal release without increasing SV number. This proposes a dual role for VACChT in vesicle filling and fusion, potentially through an interaction with synaptobrevin. It also highlights the possible importance of a polyQ domain towards the C-terminus of the *Drosophila* VACChT, as a form of VACChT with one ‘Q’ missing shows different functionality to that with the full polyQ sequence.
Chapter 5

The Giant Fiber System as a Model to Study
Insecticide Mode of Action

5.1 Introduction

My work provides convincing evidence that CASPP targets VAChtT. This conclusion has been derived from relatively low-throughput experiments that are not ideally suited to rapidly identify the mode-of-action of larger numbers of novel insecticidal compounds. A relatively simple screening technique, using *Drosophila*, would be of great benefit because of the many advantages *Drosophila* has over other insect models. For example, a suspected target protein can be overexpressed in specific tissues or cells and there are multiple RNAi lines available to knock down target transcripts (TRiP, 2015). This chapter aims to assess an electrophysiological approach using the intact adult *Drosophila* as a model to study insecticide mode of action.

The adult fly contains a well characterised neuronal pathway known as the giant fibre system (GFS) (Allen *et al.*, 2006). This pathway forms part of the escape response in which the fly jumps from the surface and flies away in response to a perceived threat, in the form of a shadow moving across the eye. This pathway consists of the giant fibre (GF), so called because of its large axonal diameter (6-8µm) that has its cell body in the head and an axon that projects into the thorax (Fig. 5.1A). The GF synapses with the tergotrochanteral motoneuron (TTMn), triggering contraction of the TTM muscle. This muscle controls extension of the mesothoracic leg, causing the fly to push itself off the surface (Fig. 5.1A). The synapse between the GF and TTMn is electrochemical, and the chemical component is cholinergic (Balgburn *et al.*, 1999; Allen and Murphey, 2007; Phelan *et al.*, 2008). The GF also synapses with the peripherally synapsing interneuron (PSI) which in turn synapses with the dorsal longitudinal muscle motoneuron (DLMn), triggering contraction in the DLM (Fig. 5.1A). The DLM controls wing depression, and is involved in flight. The synapse between the GF and PSI is electrochemical, however the PSI to DLMn synapse is a purely chemical synapse.
and is cholinergic (Fayyazuddin et al., 2006). The additional synapse between the GF and the DLM means that during an escape response, the fly first pushes off from the surface before extending the wings. The GFS can also be triggered by applying an electrical stimulus to the brain and GFS output to the muscles can be easily monitored by inserting recording electrodes into DLM 45a and the TTM (Allen and Godenschwege, 2010) (Fig. 5.1B). This technique is utilised in this chapter to characterise GFS output under insecticide treatment.

**Figure 5.1** The *Drosophila* GFS

**A.** Schematic showing neuronal connections within one side of the bilateral pathway of the GFS. The GF (red) forms mixed electrochemical synapses with the TTMn (blue) and PSI (green). The PSI forms chemical synapses with the DLMns (yellow). The NMJs are chemical synapses.  
**B.** Cartoon of GFS with neurotransmitters labelled.  
**C.** Artistic impression of the GFS within the fly. Positioning of the stimulating electrodes in the brain is shown and recording electrodes in the muscles. Adapted from Allen and Godenschwege (2010).
The GFS contains many target areas for insecticides. Within the cholinergic synapse are the targets: nAChR, AChe and VACHT. The glutamatergic NMJ between the DLMn, TTMn and their respective muscles presents other targets, with a possible mode of action of the glutamate receptor. Other targets present throughout the GFS are voltage gated sodium channels (VGSC), voltage gated potassium channels (VGKC) and GABA/glutamate-gated chloride channels (Butler et al., 1989; Cully et al., 1996; Pittendrigh et al., 1997).

The aim of this chapter is to carry out an initial screen to explore the utility of the GFS as a medium throughput approach to determine insecticide mode of action. The majority of work was carried out by applying insecticidal compounds through contact/feeding assays, but I also include data where compounds were directly injected into the body of the fly.

### 5.2 Results

Insecticidal compounds tested in the GFS system were chosen to cover a range of targets, commercial compounds with known mode of action were tested and also compounds with unconfirmed mode of action. Compounds of unknown mode of action were supplied by Syngenta and are referred to as compound A-E. Lethality to adult flies was assessed after 24 hrs of exposure and the dose range adjusted to give between 0 - 100% mortality (Fig. 5.2). Three doses that spanned this range were chosen for GFS recordings. Compound treatment was carried out in 24-well plates (see methods), this method was used because multiple assays could be carried out quickly. Control flies were treated with vehicle for 24 hrs.

Where appropriate, compounds have been grouped into target areas of: VACHT, nAChR, VGSC and NMJ. Excitatory junctional potentials (EJPs) were recorded from flight muscle DLM 45a and leg extensor muscle TTM in response to an electrical stimulus applied to the CNS (30-50 V), experiments were carried out at room temperature (18-22°C). The effect that compounds had on the GFS was tested by examining response latency, amplitude and following frequency. Response latency represents length of time between stimulus and EJP, and was measured as time from stimulus artefact to onset of response (Fig. 5.3A,C). An increase in response latency suggests a delay in transmission along the GFS network. Amplitude represents the size of the depolarisation in the muscle and was measured from the
lowest point after the stimulus artefact (but before the EJP) to the peak of the response (Fig. 5.3A,C). A change to amplitude might indicate either a change to glutamate released from the motoneuron or a change to the glutamate receptor. Following frequency measures the ability of the system to respond 1:1 with a high frequency stimulus. This was measured by applying ten trains of ten stimuli at 100 Hz and scoring successful responses out of ten (Fig. 5.3B,D). If EJPs are unable to follow 1:1 with stimulus, this could suggest that some channels (e.g. VGSC) have not returned to the state in which they may be activated again or that there is a depletion of neurotransmitter containing vesicles at the chemical synapse.
Figure 5.2  Mortality assays in adult Drosophila

Mortality assays used to obtain compound doses that span 0 – 100% mortality for use in GFS recordings. **A.** CASPP was tested at a range of 0.5 - 50 µg/ml and doses 1, 5 and 10 µg/ml were selected to be tested in GFS recordings. **B.** Compound A was tested at a range of 10 - 300 µg/ml and doses 50, 100 and 300 were selected. **C.** Compound B was tested at a range of 10 - 500 µg/ml and doses 20, 40 and 100 µg/ml were selected. **D.** Spinosad was tested in the range 2 - 32 µg/ml and concentrations 8, 16 and 32 µg/ml were selected. **E.** Compound C was tested in the range 1 - 50 µg/ml and doses 5, 10 and 50 µg/ml were chosen. **F.** Indoxacarb was applied at a range of 10 - 1000 µg/ml and did not cause increased mortality in this range. **G.** DCJW was tested at 0.5 - 8 µg/ml and doses 0.5, 2 and 4 were chosen. **H.** Deltamethrin was applied at 0.01 - 1 µg/ml and doses 0.01, 0.05 and 0.5 µg/ml were chosen. **I.** Compound D was tested in the range 10 - 1000 µg/ml and did not cause increased mortality in this range. **J.** Philanthotoxin was tested in the range 10 - 1000 µg/ml and did not cause increased mortality in this range. **K.** Compound E was tested in the range 10 - 1000 µg/ml and doses 100, 500 and 1000 µg/ml were selected. **L.** Fipronil was applied at 2 - 16 µg/ml and doses 2, 8 and 16 µg/ml were selected. N ≥ 2 samples of 10 flies. Values represent mean ± SEM.

Control (CS) flies were characterised. A response latency (RL) of 1.47 ± 0.01 ms in the DLM and 1.05 ± 0.01 ms in TTM was found (Fig. 5.3A,C), which is in good agreement with the expected range reported in the literature; DLM: 1.3-1.7 ms, TTM: 0.7-1.2 ms (Augustin *et al.*, 2011). Amplitude was 59.1 ± 1.4 mV in DLM and 22.1 ± 0.9 mV in TTM. A high level of variation in amplitude was found in both muscles and so caution should be applied in interpreting this data. Following frequency (FF) in DLM was 7.2 ± 0.2 out of 10 and in TTM was 9.4 ± 0.1 out of 10 (Fig. 5.3B,D). Both muscles are able to respond 1:1 with 100 Hz stimuli (Augustin *et al.*, 2011). Explanations for the failure in responses seen in this study could be a relatively low room temperature (minimum 18°C) or poor placement of stimulus electrodes. Control recordings were carried out regularly, interspersed with compound treated flies and therefore sample size for this group is high. The following sections describe the effect of compounds targeted at specific areas within the GFS. Significant results are reported in the text and complete data can be found in tables at the end of this chapter.
Figure 5.3  EJPs in the DLM and TTM

A. A single EJP in the DLM, illustrating amplitude and response latency (red arrows) in control. Black arrow shows stimulus artefact. B. Representative traces showing DLM following frequency test (train of 10 stimuli) demonstrating some failure (dots) of control to respond 1:1 with stimulus. C. A single EJP in the TTM, illustrating amplitude and response latency of control. D. Representative traces showing TTM following frequency.

5.2.1 The cholinergic synapse

The pathway from the GF to the TTMn contains an electrochemical synapse, whereas the pathway from the GF to DLMn contains both an electrochemical pathway (GF-PSI) and a purely chemical synapse (PSI-DLMn). Both the chemical aspect of the electrochemical synapses and the PSI-DLMn synapses are cholinergic (Fayyazuddin et al., 2006; Allen and Murphey, 2007). The electrical component of the electrochemical synapses is sufficient to maintain transmission when the cholinergic component is blocked (Allen and Murphey, 2007). Compounds that inhibit the cholinergic synapse, such as the nAChR inhibitor mecamylamine hydrochloride, cause a characteristic loss of the DLM response because of inhibition.
of the PSI-DLMn synapse but do not affect the TTM (Mejia et al., 2010). Therefore, where insecticidal compounds have a target within the cholinergic synapse this characteristic affect to the DLM but not TTM links the target to cholinergic signalling.

5.2.1.1 VAChT

The VAChT as a target of insecticides has been explored in the previous two chapters. Block of the VAChT by 5CL-CASPP (CASPP) led to a decrease in quantal release of ACh at the synapse. Here, CASPP was tested on the adult fly to provide a baseline of effect when VAChT is blocked. Two unvalidated mode of action compounds (A and B) were also tested. An overview of the results is provided in the table below and then discussed in more detail.

Table 5.1 Overview of change in DLM and TTM EJPs after compound treatment targeted at VAChT. RL = Response Latency, Amp = Amplitude, FF = Following Frequency

| Compound/ genotype | DLM | | | | TTM | | 
|-------------------|-----------------|---|---|---|---|---|---|
|                   | RL  | Amp | FF | RL  | Amp | FF | 
| CASPP             | ↑   |    | ↓  |    |    | ↓  | 
| Compound A        |    | ↓   |    |    |    |    | 
| Compound B        | ↑   |    |    |    |    |    | 
| Cha>VACHT<sup>(SYN)</sup> |    |    |    |    |    |    | 
| Cha>VACHT<sup>(SYN)</sup> + CASPP | ↑   |    |    |    |    |    | 

It was predicted that CASPP would affect the DLM and not the TTM because the neuronal pathway to this DLM contains a cholinergic synapse, with no electrical component (which maintains synaptic transmission when the chemical component is blocked (Allen and Murphey, 2007)). It was not expected that this compound would affect the TTM because all synapses in this pathway are electrochemical (with the exception of the NMJ which is glutamatergic). Concentrations tested were 1, 5 and 10 µg/ml (Fig. 5.2A). DLM response latency was significantly increased at 5 and 10 µg/ml (1 µg/ml: 1.62 ± 0.05 ms, P = 0.2; 5 µg/ml: 1.76 ± 0.07 ms, P = 0.004; 10 µg/ml: 2.06 ± 0.1 ms, P < 0.001, vs. control, Fig. 5.4A,B). DLM amplitude was not affected at any concentration (Fig. 5.4D). DLM following frequency was significantly decreased at all concentrations (1 µg/ml: 3.0 ± 0.5 out of 10, P < 0.001; 5 µg/ml: 0.8 ± 0.2 out of 10, P < 0.001; 10 µg/ml: 1.0 ± 0.6 out of 10, P < 0.001, vs. ...
control, Fig. 5.4F, Table 5.6). Response latency and amplitude were unchanged in the TTM (Fig. 5.4C,E, Table 5.6). TTM following frequency was unexpectedly decreased at 10 µg/ml CASPP (7.2 ± 1.1 out of 10, \( P = 0.01 \), vs. control). The increase in response latency and decrease in following frequency of the DLM is consistent with disruption of the cholinergic synapse between the PSI and DLM, however, the less pronounced effects on TTM at the highest dose may indicate an additional mode of action of CASPP.

Two compounds with unvalidated mode of action, but predicted to target VAChT (private communication, Syngenta) were tested; compound A and B. Compound A had low solubility, therefore this compound gave a high level of variability in mortality. This compound also caused paralysis that was difficult to distinguish from death. Compound A was applied at 50, 100 and 300 µg/ml (Fig. 5.2B). DLM response latency was not significantly changed (Fig. 5.2B, Table 5.6). DLM amplitude was significantly decreased at 100 µg/ml (37.1 ± 8.9 mV, \( P = 0.002 \), vs. control, Fig. 5.2D) but was not affected at 50 and 300 µg/ml. This result may be caused by the high variation found in EJP amplitudes. DLM following frequency was significantly decreased at all concentrations of compound A tested (50 µg/ml: 1.5 ± 0.2 out of 10, \( P < 0.001 \); 100 µg/ml: 1.2 ± 0.4 out of 10, \( P < 0.001 \); 300 µg/ml: 0.8 ± 0.2 out of 10, \( P < 0.001 \), vs. control, Fig. 5.4F, Table 5.6). TTM response latency and amplitude was not changed at any concentration (Fig. 5.2C,E, Table 5.6). TTM following frequency was decreased and found to be significant at 50 and 300 µg/ml (50 µg/ml: 6.2 ± 1.7 out of 10, \( P = 0.01 \); 100 µg/ml: 7.8 ± 2.3 out of 10, \( P = 0.5 \); 300 µg/ml: 6.0 ± 1.9 out of 10, \( P = 0.003 \), vs. control, Fig. 5.4G, Table 5.6). These results, which mirror those seen with CASPP indicate that compound A has an inhibitory effect at the cholinergic synapse, particularly demonstrated by a reduced following frequency in the DLM. However, reduced following frequency in the TTM may indicate additional mode of action.
Fig. 5.4

A  

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<th>DLM</th>
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<tr>
<td>Control</td>
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<td>CASPP (10µg/ml)</td>
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<td>Compound A (300µg/ml)</td>
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<td>Compound B (100µg/ml)</td>
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B  

B DLM Response latency

C  

C TTM Response latency

D  

D DLM Amplitude

E  

E TTM Amplitude

F  

F DLM Following frequency

G  

G TTM Following frequency
**Figure 5.4** DLM and TTM EJPs after compound treatment targeted at VACHT

A. Representative traces illustrating response latency and following frequency in the DLM and TTM after treatment with CASPP (10 µg/ml), compound A (300 µg/ml) and compound B (100 µg/ml). Dots indicate failure of EJP in response to stimulation. **B.** DLM response latency is not significantly altered in flies treated with 1 µg/ml CASPP, but is increased in those treated with 5 and 10 µg/ml. Compound A caused no significant effect on DLM response latency at any concentration. Compound B did not affect DLM response latency at 20 and 40 µg/ml but caused a significant increase at 100 µg/ml. **C.** TTM response latency was unchanged in flies treated with CASPP, compound A or compound B. **D.** CASPP did not affect DLM amplitude at any concentration. Compound A caused a significant decrease in amplitude of DLM at 100 µg/ml but showed no effect at 50 or 300 µg/ml. Compound B caused a significant decrease in amplitude of DLM at 40 µg/ml but showed no effect at 20 or 100 µg/ml. **E.** TTM amplitude was unaffected by CASPP, compound A or compound B. **F.** DLM following frequency was significantly reduced by CASPP, compound A and compound B at all concentrations tested. **G.** TTM following frequency was not significantly reduced in flies treated with 1 and 5 µg/ml CASPP, but was significantly reduced in flies treated with 10 µg/ml. TTM following frequency was significantly decreased in flies treated with 50 and 300 µg/ml compound A but was not significantly changed at 100 µg/ml. TTM following frequency was not significantly changed in flies treated with compound B. Numbers within columns represent N. Values are mean ± SEM.

Compound B was also found to cause a high level of paralysis, therefore doses were chosen that represented high and low paralysis as well as mortality. Compound B was applied at 20, 40 and 100 µg/ml (Fig. 5.2C). DLM response latency was significantly increased at 100 µg/ml (1.72 ± 0.2, P = 0.006, vs control, Fig. 5.4A). DLM amplitude was significantly decreased at 40 µg/ml (37.4 ± 2.5, P = 0.004, vs. control, Fig. 5.4D) but not at 20 or 100 µg/ml. It is likely that this result was due to low sample size and high variability. DLM following frequency was significantly decreased at all concentrations of compound B (20 µg/ml: 1.6 ± 0.5 out of 10, P < 0.001; 40 µg/ml: 1.3 ± 0.5 out of 10, P < 0.001; 100 µg/ml: 0.9 ± 0.2 out of 10, P < 0.001, vs. control, Fig. 5.4F, Table 5.6). TTM response latency, amplitude and following frequency was not significantly changed (Fig. 5.4A,C,E,G, Table 5.6). Although sample size was low due to time constraints, the effect of compound B in reducing following frequency of the DLM suggests that it is also acting at cholinergic synapses, consistent with the hypothesis that VACHT is the target.
In previous chapters, Drosophila that overexpress VACht in cholinergic neurons (cha>VAChT+(SYN)) were used to demonstrate that the VACht is a target of CASPP. Larvae were ~3-fold less sensitive to CASPP in mortality assays and showed rescue of ACh release in electrophysiological recordings (chapter 3, Fig. 3.6). To test if expression of VACht+(SYN) in adult flies produced the same predicted level of resistance to CASPP, cha>VAChT+(SYN) were overexpressed using cha driver which expresses in the cholinergic GF and PSI interneurons. These flies showed only ~10% mortality at 10 μg/ml CASPP treatment. Cha>VAChT+(SYN) EJPs were not different to control flies (control was parental transgene controls and CS) (Fig. 5.5, Table 5.6). These flies were treated with CASPP at 1, 5 and 10 μg/ml. DLM response latency was significantly increased at 5 and 10 μg/ml (5 μg/ml: 2.32 ± 0.6 ms, P < 0.001; 10 μg/ml: 2.26 ± 0.4, P < 0.001, vs. control, Fig. 5.5A,B, Table 5.6). Unexpectedly, the response latency in cha>VAChT+(SYN) flies was higher than control treated with CASPP at the same concentrations (Fig. 5.4B). DLM amplitude was unchanged (Fig. 5.5D). DLM following frequency was significantly decreased at all concentrations tested (1 μg/ml: 3.7 ± 0.9 out of 10, P < 0.001; 5 μg/ml: 0.4 ± 0.2 out of 10, P < 0.001; 10 μg/ml: 0.2 ± 0.1 out of 10, P < 0.001, vs. control, Fig. 5.5F, Table 5.6). TTM response latency and amplitude was not changed (Fig. 5.5A,C,E, Table 5.6). However, TTM following frequency was significantly decreased at all concentrations of CASPP (1 μg/ml: 6.4 ± 0.7 out of 10, P < 0.001; 5 μg/ml: 5.7 ± 1.8 out of 10, P < 0.001; 10 μg/ml: 6.6 ± 1.7 out of 10, P = 0.006, vs. control, Fig. 5.5G, Table 5.6). This result is unexpected because, in wild-type flies, CASPP only affected following frequency in the TTM at 10 μg/ml and may suggest that cha>VAChT+(SYN) flies are more susceptible to this secondary effect of CASPP. Cha>VAChT+(SYN) flies are certainly more resistant to CASPP in mortality assays but it may be that overexpression of the VACht is not sufficient to rescue the effects of CASPP in the GFS under the artificial stimulation applied during GFS recordings. These results demonstrate that the GFS is able to indicate a compound target at the cholinergic synapse and also demonstrates that the effect of compounds may be more pronounced under conditions of electrical stimulation. However, without additional characterisation, the results gained, at least for VACht-targeting compounds are mixed.
Fig. 5.5

**A**

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<td>CASPP</td>
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<td>n.s</td>
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**DLM amplitude**

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**F**

**DLM Following frequency**

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**G**

**TTM Following frequency**

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<th>Cha&gt;VAChT + 1µg/ml</th>
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<td>11</td>
<td>8</td>
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</tr>
<tr>
<td>CASPP</td>
<td>n.s</td>
<td>n.s</td>
<td>***</td>
<td>***</td>
<td>n.s</td>
<td>n.s</td>
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</tbody>
</table>

**Legend:**

- DLM: Direct Lateral Motor neuron
- TTM: Thalamo-thalamic nucleus motor neuron
- CASPP: Caudate-putamen
- Cha: Choline acetyltransferase
- VAChT: Vesicular acetylcholine transporter
- n.s: Not significant
- ***: Significant at the 0.001 level
- **: Significant at the 0.01 level
- mV: Millivolt

**Note:** The diagrams and figures are not included in the text representation.
Figure 5.5  DLM and TTM EJPs in flies that overexpress VACht in cholinergic neurons

A. Representative traces illustrating response latency and following frequency in the DLM and TTM in flies that overexpress VACht in cholinergic neurons (cha>VAChT\textsuperscript{(SYN)}) and the same genotypes after CASPP treatment (10 µg/ml). Dots indicate failure of EJP in response to stimulation. B. DLM response latency is not significantly changed in cha>VAChT\textsuperscript{(SYN)} flies or in flies treated with 1 µg/ml CASPP. DLM response latency is significantly increased in cha>VAChT\textsuperscript{(SYN)} flies treated with 5 and 10 µg/ml CASPP. C. TTM response latency was unchanged in untreated or CASPP treated cha>VAChT\textsuperscript{(SYN)} flies. D. DLM amplitude was unchanged in untreated or CASPP treated cha>VAChT\textsuperscript{(SYN)} flies. E. TTM amplitude was unchanged in untreated or CASPP treated cha>VAChT\textsuperscript{(SYN)} flies. F. DLM following frequency was not significantly changed in cha>VAChT\textsuperscript{(SYN)} flies but was significantly reduced in flies treated with 1, 5 and 10 µg/ml CASPP. G. TTM following frequency was not changed in cha>VAChT\textsuperscript{(SYN)} flies but was significantly reduced in flies treated with 1, 5 and 10 µg/ml CASPP. Numbers within columns represent N. Values are mean ± SEM.

5.2.1.2 nAChR

The insect nAChR has long been a target of insecticides and species difference in subunits makes it a good target for the design of species specific insecticides (Jones and Sattelle, 2010). In Drosophila the nAChR consists of 5 subunits from a possible 10. It is not known which subunits make up the nAChR at synapses in the GFS but Dα7 has been shown to be of importance at the PSI-DLMn synapse (Fayyazuddin et al., 2006; Mejia et al., 2013). The specific expression of subunits in this system will effect which insecticides are effective and so not all insecticides that target the nAChR may be detectable using this method. In this study two subunit null fly lines were tested as well as the commercial insecticide spinosad and an unvalidated compound, compound C. Results are summarised in Table 5.2.
Initially, two available subunit null mutants were tested: Dα7 and Dα6, to assess their importance in the GFS. In null Dα7 flies only 3 / 8 flies produced an EJP in the DLM in response to stimuli. DLM response latency was significantly increased (5.17 ± 0.4 ms, $P < 0.001$, vs. control, Fig. 5.6A,B, Table 5.7) and there was no effect on amplitude (Fig. 5.6D). DLM following frequency was significantly decreased (0.4 ± 0.2 out of 10, $P < 0.001$). TTM response latency, amplitude and following frequency were not different to control (Fig. 5.6A,C,E,G, Table 5.7). This indicates that, as expected, the electrical component of the GF to TTMn synapse is sufficient to compensate for a lack of Dα7. These results are in agreement with the literature (Fayyazuddin et al. 2006; Mejia et al., 2013).

Flies null for Dα6 did not show any change to DLM or TTM (Fig. 5.6, Table 5.7), suggesting that Dα6 is not expressed, at least at high levels, at the PSI to DLMn synapse. Flies null for Dα6 have developed without this subunit and may have compensated for its absence by substitution with a similar subunit. To test this, flies were treated with spinosad, an allosteric modulator of the nAChR. Spinosad is known to target Dα6 because null Dα6 flies are ~1000-fold more resistant (Perry et al., 2007). Spinosad was tested at 8, 16 and 32 µg/ml (Fig. 5.2D). DLM response latency was significantly increased at 32 µg/ml (1.66 ± 0.1 ms, $P = 0.02$, vs. control, Fig. 5.6B, Table 5.7). DLM amplitude and following frequency were not different to control (Fig. 5.6D,F, Table 5.7). TTM response latency, amplitude and following frequency were unaffected (Fig. 5.6C,E,G, Table 5.7). The, small, affect to the DLM at the highest dose tested is consistent with the presence of Dα6 at the PSI to DLMn synapse at low levels or may indicate binding of spinosad to other subunits.

### Table 5.2  Overview of change in DLM and TTM EJPs after compound treatment targeted at nAChR. RL = Response Latency, Amp = Amplitude, FF = Following Frequency

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<tr>
<td>Null Dα6</td>
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<td>─</td>
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<tr>
<td>Spinosad</td>
<td>↑</td>
<td>─</td>
</tr>
<tr>
<td>Compound C</td>
<td>↑</td>
<td>─</td>
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</table>
Fig. 5.6

A  DLM  TTM
  control
  Null Da7
  Null Da6
  Spinosad 32µg/ml
  Compound D 50µg/ml

B  DLM Response latency
  C TTM Response latency

D  DLM Amplitude
  E TTM Amplitude

F  DLM Following frequency
  G TTM Following frequency

***  ***  n.s  n.s  n.s  n.s
7  3  6  6  6  7
7  7  7  7  7  7

spinosad  Compound C
Figure 5.6  DLM and TTM EJPs in nAChR subunit null flies and after nAChR targeted compound treatment

A. Representative traces illustrating response latency and following frequency in the DLM and TTM in flies null for Da7 and Da6, and control wild-type flies treated with spinosad (32 µg/ml) and compound D (1 and 2, 500 µg/ml). Dots indicate failure of EJP in response to stimulation. B. DLM response latency is significantly increased in null Da7 flies but is not affected in flies null for Da6. Spinosad had no significant effect on DLM response latency at 8 and 16 µg/ml but caused a significant increase at 32 µg/ml. Compound D caused a significant increase in DLM response latency at 5, 10 and 50 µg/ml. C. TTM response latency was unchanged in null Da7, null Da6 and in flies treated with spinosad or compound D. D. DLM amplitude was unchanged in null Da7, null Da6 and in flies treated with spinosad or compound D. E. TTM amplitude was unchanged in null Da7, null Da6 and in flies treated with spinosad or compound D. F. DLM following frequency was significantly reduced in null Da7 flies but was not significantly changed in null Da6 or in flies treated with spinosad or compound D. G. TTM following frequency was not significantly reduced in null Da7, null Da6 flies or flies treated with spinosad. TTM following frequency was unchanged in flies treated with 5 or 10 µg/ml of compound D but was significantly reduced at 50 µg/ml. Numbers within columns represent N. Values are mean ± SEM.

A compound with unconfirmed mode of action, but to which null Da6 flies also show resistance (private communication, Syngenta) was tested. The compound, termed compound C was tested at 5, 10 and 50 µg/ml (Fig. 5.2E). DLM response latency was significantly increased at all concentrations (5 µg/ml: 1.77 ± 0.2 ms, P < 0.001; 10 µg/ml: 1.68 ± 0.05 ms, P = 0.05; 50 µg/ml: 1.76 ± 0.1 ms, P < 0.001, vs. control, Fig. 5.6A,F, Table 5.7). DLM amplitude and following frequency were not different to control at any concentration (Fig. 5.6D,F, Table 5.7). The shape of the DLM EJP in compound C treated flies was also observed to be an unusual shape (Fig. 5.5A) which was witnessed most often at 50 µg/ml. TTM response latency and amplitude were not changed (Fig. 5.6C,E, Table 5.7). TTM following frequency was significantly decreased at 50 µg/ml (6.9 ± 1.3 out of 10, P = 0.001, vs. control, Fig. 5.6G). The similarity in response to spinosad suggests that compound C primarily acts at a cholinergic synapse, however because increased response latency in DLM was found at all test concentrations, compound C may not be as specific in its target as spinosad. The effect on TTM following frequency may suggest a second mode of action but a higher sample size is required to confirm this. These results show that the GFS can possibly identify allosteric modulators of the nAChR as targeting the
cholinergic synapse by their effect on DLM response latency but not following frequency.

### 5.2.2 VGSC

The VGSC is essential for the transmission of action potentials and is present throughout the nervous system. It was predicted that a compound that targets the VGSC would affect both the DLM and TTM because both pathways rely on the VGSC. An insecticide known to target the insect VGSC is indoxacarb. This compound is metabolised by the insect to the active compound decarbomethoxylated JW062 (DCJW) and it is DCJW that binds to the VGSC (Wing et al., 1998). Both indoxacarb and DCJW were tested as well as the VGSC agonist deltamethrin and compound D. Results are summarised in table 5.3.

**Table 5.3** Overview of change in DLM and TTM EJPs after compound treatment targeted at VGSC. RL = Response Latency, Amp = Amplitude, FF = Following Frequency

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<td>Compound D</td>
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Flies were treated with indoxacarb at concentrations up to 1000 μg/ml but no mortality was reported after 24 hrs (Fig. 5.2F). After 72 hrs ~ 30% of flies at 1000 μg/ml were dead (control showed 0% mortality). The remaining living flies were recorded from and showed a decrease in following frequency in the DLM (5.2 ± 1.1 out of 10, P = 0.03) but no effect on response latency in either muscle or following frequency in TTM (control flies at 72 hrs were not different to 24 hr) (Fig. 5.7, Table 5.8). This outcome is unexpected because an antagonistic effect on the VGSC would be expected to affect nerve transmission to both muscles. The reasons for this result are unknown but may, conceivably, be due to method of compound application, metabolism and/or time of exposure. In order to achieve more effective inhibition of the VGSC, the metabolite DCJW was tested and found to cause mortality within 24 hrs at concentrations <10 μg/ml (Fig. 5.2G). Concentrations used were 0.5, 2 and 4 μg/ml. Surprisingly, DCJW only had a significant effect on EJPs at 0.5 μg/ml (<10% mortality). DLM and TTM response latency was increased...
compared to control at 0.5 μg/ml (DLM: 1.79 ± 0.2 ms, P = 0.002; TTM: 1.27 ± 0.1 ms, P = 0.002, Fig. 5.7A-C, Table 5.8). Amplitude in both muscles was not affected (Fig. 5.7D,E, Table 5.8). Following frequency was significantly decreased in DLM and TTM at 0.5 μg/ml (DLM: 4.2 ± 1.0 out of 10, P = 0.007; TTM: 5.1 ± 1.2 out of 10, P < 0.001, Fig. 5.7F,G, Table 5.8). An effect on both muscles is consistent with the sodium channel target, however the observed effect only at a low dose was not expected and will be discussed in section 5.3. These results may expose a flaw in the experimental design, in that flies which have survived high doses of insecticide are those that are more resistant. However, flies treated at 4 μg/ml appeared almost completely paralysed and only moved in response to physical stimuli (i.e. touched with forceps) and so did not appear to be resistant to DCJW.

Deltamethrin is a pyrethroid insecticide that binds to the VGSC and modifies gating kinetics, leading to prolonged Na+ currents which eventually leads to paralysis (Chinn and Narahashi, 1986; Vais et al., 2003; Davies et al., 2007). Flies exposed to deltamethrin exhibit shaking behaviour due to increased excitation of the nervous system followed by paralysis. So far the GFS has been shown to be effective in demonstrating the mode of action of compounds that have an antagonistic mode of action. The ability to distinguish an effect of excitatory compounds would provide additional advantage. Deltamethrin was extremely potent and was applied at 0.01, 0.05 and 0.5 μg/ml (Fig. 5.2H). No effect on DLM or TTM was found at any concentration (Fig. 5.7, Table 5.8). This result suggests that the GFS is not a good system to study mode of action of insecticides which have an excitatory effect on the nervous system.

An unconfirmed mode of action compound, Compound E, proposed to have antagonistic activity on VGSC and weak antagonistic effect on VACHT (private communication, Syngenta) was tested. This compound did not cause mortality at concentrations up to 1000 μg/ml (Fig5.2l). When applied at 4000 μg/ml, this compound was not lethal at 24 hrs. Flies were recorded from after 24 hrs at this dose to test for possible sub-lethal effects. DLM response latency and amplitude were not changed (Fig. 5.7A,B,D, Table 5.8). DLM following frequency was significantly reduced (4.1 ± 1.3 out of 10, P = 0.002, Fig. 5.7F, Table 5.8). TTM response latency, amplitude and following frequency was unchanged (Fig. 5.7C,E,G). This suggests that, in Drosophila, this compound did not affect the VGSC but possibly targeted the cholinergic synapse, leading to an effect on DLM.
Fig. 5.7

A

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B

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D

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F

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<td></td>
<td><img src="image29" alt="Graph" /></td>
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</table>
Figure 5.7 DLM and TTM EJPs after compound treatment targeted at VGSC

A. Representative traces illustrating response latency and following frequency in the DLM and TTM in flies treated with indoxacarb (72 hrs; 1000 µg/ml), DCJW (0.5 and 4 µg/ml), deltamethrin (0.5 µg/ml) and compound E (4000 µg/ml). Dots indicate failure of EJP in response to stimulation. B. DLM response latency was not significantly changed in flies treated with indoxacarb for 72 hrs. DLM response latency was significantly increased in flies treated with 0.5 µg/ml but was not changed at 2 or 4 µg/ml. Deltamethrin and compound E did not affect DLM response latency. C. TTM response latency was unchanged in flies treated with indoxacarb. TTM response latency was increased in flies treated with 0.5 µg/ml but was not changed at 2 or 4 µg/ml. Deltamethrin and compound E did not affect TTM response latency. D. DLM amplitude was unchanged in flies treated with indoxacarb, DCJW, deltamethrin or compound E. E. TTM amplitude was unchanged in flies treated with indoxacarb, DCJW, deltamethrin or compound E. F. DLM following frequency was significantly reduced in flies treated with 1000 µg/ml indoxacarb. DCJW significantly decreased DLM following frequency at 0.5 µg/ml but had no effect at 2 or 4 µg/ml. Deltamethrin did not affect DLM following frequency. Compound E significantly reduced DLM following frequency at 4000 µg/ml. G. TTM following frequency was not significantly reduced in flies treated with indoxacarb. DCJW significantly decreased TTM following frequency at 0.5 µg/ml but had no effect at 2 or 4 µg/ml. Deltamethrin and compound E had no significant effect on TTM following frequency. Numbers within columns represent N. Values are mean ± SEM.

5.2.3 NMJ

A potential insecticidal target that is most predominant at the insect NMJ is the glutamate gated cation channel (GluR) (Johansen et al., 1989; Eldefrawi et al., 1993). Currently, there are no commercial insecticides with this mode of action (IRAC, 2015). The ionotropic GluRs open when glutamate is bound, leading to an EJP in the muscle. It was predicted that compounds that target GluRs would affect both the DLM and TTM. The glutamate receptor antagonist philanthotoxin and an unvalidated mode of action compound, compound E were tested and table 5.4 summarises the results.
Table 5.4 Overview of change in DLM and TTM EJPs after compound treatment targeted at the NMJ. RL = Response Latency, Amp = Amplitude, FF = Following Frequency

<table>
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<tr>
<th>Compound/genotype</th>
<th>DLM</th>
<th>TTM</th>
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<tbody>
<tr>
<td>Philanthotoxin</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>Compound E</td>
<td>↓</td>
<td>↓</td>
</tr>
</tbody>
</table>

The wasp toxin philanthotoxin is an antagonist of the glutamate receptor (Eldefrawi et al., 1998; Frank et al., 2006). Philanthotoxin did not cause increased mortality in assays up to 1000 µg/ml (Fig. 5.2J) and so applied dose was increased to 3000 µg/ml. Flies treated at this dose showed no increased mortality after 24 hrs and were then recorded from to test for sub-lethal effects of the toxin. DLM and TTM response latency was significantly increased compared to control (DLM: 1.65 ± 0.06 ms, P < 0.001; TTM: 1.14 ± 0.04 ms, P = 0.03, Fig. 5.8A-C, Table 5.9). Amplitude and following frequency was not affected in either muscle (Fig. 5.8D-G, Table 5.9). The increased response latency in both muscles would be consistent with a target at the NMJ, however it might be expected that EJP amplitude would be reduced if the GluR was inhibited and response latencies were still within the normal range expected at DLM and TTM (Augustin et al., 2011). This toxin may have had a weakened effect due to instability of the compound over the time period of the assays.

A compound of unknown mode of action, but for which there is evidence of a target site at the housefly NMJ (private communication, Syngenta) was also tested. Compound E was applied at concentrations 100, 500 and 1000 µg/ml (Fig. 5.2K). DLM response latency and amplitude was not different to control (Fig. 5.8A,B,D, Table 5.9). DLM following frequency was significantly reduced at 1000 µg/ml (3.5 ± 0.9 out of 10, P < 0.001, vs. control, Fig. 5.8E). TTM response latency and amplitude was unchanged and following frequency was not significantly different to control but showed a trend to decreased events (Fig. 5.8C,E,G, Table 5.9). This result does not contradict the NMJ as a target but would require further experiments because the TTM following frequency was not significantly decreased. This experiment also highlights a difference between these two compounds targeted at the NMJ. Philanthotoxin causes an increase in response latency with no change to following frequency and compound E causes a decrease in following frequency with no effect on response latency. This may indicate different modes of action within the NMJ.
Fig. 5.8

**A**

<table>
<thead>
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**B**

**DLM Response latency**

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**C**

**TTM Response latency**

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**D**

**DLM Amplitude**

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**E**

**TTM Amplitude**

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**F**

**DLM Following frequency**

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<th>500µg/ml</th>
<th>1000µg/ml</th>
<th>Compound E 1000µg/ml</th>
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**G**

**TTM Following frequency**

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<th>500µg/ml</th>
<th>1000µg/ml</th>
<th>Compound E 1000µg/ml</th>
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<td><img src="image41" alt="TTM frequency 1000µg/ml" /></td>
<td><img src="image42" alt="TTM frequency compound" /></td>
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</table>
**Figure 5.8** DLM and TTM EJPs after compound treatment targeted at glutamatergic NMJ

A. Representative traces illustrating response latency and following frequency in the DLM and TTM in flies treated with philanthotoxin (3000 µg/ml), and compound E (1000 µg/ml). B. Philanthotoxin caused a significant increase in response latency in the DLM. Compound E did not affect DLM response latency at any concentration tested. C. TTM response latency was increased in flies treated with philanthotoxin. Compound E did not affect TTM response latency. D. DLM amplitude was not affected by philanthotoxin or compound E. E. TTM amplitude was not affected by philanthotoxin or compound E. F. DLM following frequency was not changed by philanthotoxin. Compound E did not affect DLM following frequency at 100 or 500 µg/ml but significantly reduced following frequency at 1000 µg/ml. G. TTM following frequency was not significantly decreased by philanthotoxin or compound E. Numbers within columns represent N. Values are mean ± SEM.

### 5.2.4 GABA-gated chloride channel

GABA-gated chloride channels found in the insect CNS (Harrison *et al*., 1996) are activated by GABA and have an inhibitory action by hyperpolarising the cell (Lee *et al*., 2003). Fipronil is an antagonist of GABA-gated chloride channels, and is also reported to target glutamate-gated chloride channels (Zhao *et al*., 2005), and would therefore be expected to increase excitation in the nervous system. A summary of the findings using fipronil is provided in table 5.5 below.

<p>| Table 5.5 Overview of change in DLM and TTM EJPs after compound treatment targeted at the GABA-gated chloride channel. RL = Response Latency, Amp = Amplitude, FF = Following Frequency |</p>
<table>
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<tr>
<th>Compound/genotype</th>
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</thead>
<tbody>
<tr>
<td>Fipronil</td>
<td>RL</td>
<td>Amp</td>
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</table>

Fipronil was tested at 2, 8 and 16 µg/ml (Fig. 5.2L). DLM response latency, amplitude and following frequency was not different to control (Fig. 5.9, Table 5.10). TTM response latency and amplitude was unchanged (Fig. 5.9C,E). TTM following frequency was significantly decreased compared to control at 2 and 8 µg/ml (2 µg/ml: 7.0 ± 1.7 out of 10, P = 0.02; 8 µg/ml: 6.7 ± 1.7 out of 10, P = 0.007, Fig. 5.9G, Table 5.10). TTM following frequency at 16 µg/ml was not significantly different to control, the surviving flies at this dose may be resistant to fipronil. However, it is unexpected that a compound which inhibits the pathway to the TTM
should not influence the DLM. This is perhaps another example demonstrating that the GFS is unable to identify excitatory compounds.

Fig. 5.9

**A** DLM and TTM

**B** DLM Response latency

**C** TTM Response latency

**D** DLM Amplitude

**E** TTM Amplitude

**F** DLM Following frequency

**G** TTM Following frequency
5.2.5 Thoracic stimulation to bypass the chemical synapse

I show above that a compound which targets the cholinergic synapse generally affects the response in the DLM muscle (in particular reducing the following frequency). Confirmation of the cholinergic synapse as the target can be carried out by placing the stimulating electrodes into the thorax, just posterior to the head. A sufficient stimulus applied here will directly stimulate the motoneurons, bypassing any inhibition of the cholinergic PSI to DLM synapse. In control flies when the stimulus is applied directly to the thorax, response latency is <1 ms in both DLM and TTM muscles (DLM: 0.76 ± 0.09 ms; TTM: 0.62 ± 0.01 ms, Fig. 5.10A,B), which is consistent with direct motoneuron stimulation. Following frequency was also slightly improved in both muscles (DLM: 9.0 ± 1.0 out of 10; TTM: 10.0 ± 0.0 out of 10). This method was tested in flies null for the Dα7 subunit of the nAChR. Stimulation of the GFS via the head (i.e. as used above) shows increased response latency and decreased following frequency in the DLM (Fig. 5.10E,G). When the stimulus is applied to the thorax, response latency and following frequency are no longer different to control (response latency: 0.77 ± 0.1 ms, P = 1.0; following frequency: 8.1 ± 1.6 out of 10, P = 1.0, vs. control, control was untreated with thoracic stimulation). Amplitude was not measured after thoracic stimulation because the EJP was often obscured by the stimulus artefact (Fig. 5.10A,B). As expected, TTM response latency and following frequency in null Dα7 flies were not different to control (response latency: 0.78 ± 0.02 ms, P = 0.2; following frequency: 10.0 ± 0.0 out of 10, P = 1.0, Fig. 5.10F,H). Therefore a compound with a target at the
cholinergic synapse would be expected to affect the DLM but not TTM response during head stimulation but have no effect during thoracic stimulation.

Thoracic stimulation as confirmation of target was also tested in flies which had been treated with 10 µg/ml CASPP, a compound that targets the VAChT. Flies treated with this compound showed increased response latency in the DLM and decreased following frequency in both the DLM and TTM using head stimulation (Fig. 5.10G,H). Thoracic stimulation produced a following frequency that was not different to control in either muscle (DLM: 8.5 ± 1.4 out of 10, P = 1.0; TTM: 9.7 ± 0.3 out of 10, P = 0.6, vs. control, Fig. 5.10) but response latency in the DLM was still significantly higher in CASPP treated flies compared to control (0.88 ± 0.02 ms, P = 0.04). TTM response latency was not different to control (0.62 ± 0.07 ms, P = 1.0). The ability to apply stimulation to the thorax and bypass the cholinergic synapse gives another advantage to the GFS as a tool to identify a cholinergic mode of action.
Fig. 5.10

A  DLM response latency
Head stimulation  Thoracic stimulation

B  TTM response latency
Head stimulation  Thoracic stimulation

C  DLM following frequency
(1) Head stimulation
(2) Head stimulation (CASPP 10µg/ml)
(3) Thoracic stimulation (CASPP 10µg/ml)

D  TTM following frequency
Head stimulation  Thoracic stimulation

E  DLM Response latency
n.s  

F  TTM Response latency
n.s

G  DLM Following frequency
n.s

H  TTM Following frequency
n.s

(1) Head stimulation
(2) Head stimulation (CASPP 10µg/ml)
(3) Thoracic stimulation (CASPP 10µg/ml)
Figure 5.10  DLM and TTM EJPs during thoracic stimulation

A. Trace representing DLM response latency in control during head and thoracic stimulation.
B. Trace representing TTM response latency in control during head and thoracic stimulation.
C. DLM following frequency in control flies during head stimulation (1), CASPP (10 µg/ml) treated flies during head stimulation (2) and CASPP treated flies during thoracic stimulation (3).
D. TTM following frequency in control flies during head stimulation and thoracic stimulation.
E. Thoracic stimulation reduces response latency in null Dα7 flies to control levels. Thoracic stimulation reduced response latency in CASPP (10 µg/ml) treated flies but latency is still significantly longer than control flies.
F. TTM response latency was not different to control in null Dα7 flies or those treated with CASPP during head or thoracic stimulation.
G. DLM following frequency was increased in null Dα7 and CASPP treated flies to control levels during thoracic stimulation.
H. TTM thoracic stimulation was not different to control in null Dα7 or CASPP treated flies during thoracic stimulation. Numbers within columns represent N. Values are mean ± SEM.

5.2.6 Compound injection

A different method of compound application was trialled. Drosophila has an open circulatory system that allows fast dispersion of compound if it is injected directly into the haemolymph (Mejia et al., 2010, 2012). Compound was injected by pushing an injection needle through a ‘soft spot’ on the flies head, located in the middle of the three ocelli (Mejia et al., 2010). Compounds were injected at concentrations observed to show effects in other electrophysiological assays (private communication, Syngenta). To monitor the effect on EJPs, a train of 10 stimuli at 100 Hz was applied every 30 seconds from 1 minute before compound injection. Methyllycaconitine citrate (MLA), a compound known to inhibit mammalian Dα7 and previously shown to inhibit the PSI to DLMn pathway (Holladay et al., 1997; Mejia et al., 2012) was injected at 7 (n = 1) and 70 µg/ml (n = 1) at a volume of 9.2 nl. In both cases DLM following frequency was reduced to 1/10 almost instantly and TTM remained unaffected (Fig. 5.11B). Flies injected with saline only, continued to have normal responses in DLM and TTM for the full 15 minutes monitored (Fig. 5.11A). The nAChR agonist imidacloprid (Matsuda et al., 2001) was also tested at 9.2 nl of 3 µg/ml (n = 1), no change was observed up to 15 minutes after injection (Fig. 5.11C).
Fig. 5.11

Before injection

0 minutes

Time after injection

5 minutes

10 minutes

15 minutes

A

DLM

TTM

saline

(46 nl)

B

MLA

7 µg/ml (9.2 nl)

C

imidacloprid

3 µg/ml (9.2 nl)
Figure 5.11  DLM and TTM EJP following frequency after injection of compounds that target nAChR

A. Injection of 46 nl of saline does not affect DLM or TTM during 15 minutes monitored. B. Injection of 9.2 nl of 7 µg/ml MLA reduces DLM response to 1/10 immediately after injection but does not affect the TTM. C. Injection of 9.2 nl of imidacloprid does not affect the DLM or TTM for the 15 minutes responses were monitored. Dots represent failed EJPs.

The sodium channel agonist deltamethrin was injected at 36.8 nl of 50 µg/ml (n = 1) and caused failure in the DLM after 5 minutes and failure of TTM by 15 minutes. When the same volume of a 10% DMSO solution (vehicle) was injected both muscles were still able to respond to stimuli after 15 minutes (Fig. 5.12A). This result is different to the feeding / contact assay application of deltamethrin where no change in either muscle was found. Although a loss of response in both muscles was found, this does not indicate the excitatory mode of action of deltamethrin.

A second compound also tested in feeding/contact assays was the VAChT inhibitor CASPP. 50 µg/ml of CASPP was injected at 4 volumes: 9.2, 18.4, 27.6 and 38.8 nl (n = 1 for each). Failure was observed in the DLM at 18.4 and 27.6 nl after ~10 minutes. 9.2 and 38.8 nl had no effect after 15 minutes. 9.2 nl of 500 µg/ml was injected in two trials, one trial caused failure of the DLM at 5 minutes but the second caused no effect up to 15 minutes after injection (Fig. 5.12B). When CASPP was injected at 46 nl of 500 µg/ml, no effect was seen up to 15 minutes. No effect on the TTM was found in any of the trials. This variability in results suggests that this method of compound application may not give consistent results.

This method of compound application gave clear results for nAChR inhibitor MLA and VGSC agonist deltamethrin. However, results using CASPP were variable. Other problems encountered with this method were disruption of recording electrodes on insertion of the injection needle and leak of compound from the injection needle when it was pushed through the cuticle. Therefore, this method required further optimisation that time did not allow during the course of this project.
Fig. 5.12

<table>
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<th>Time after injection</th>
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<th>5 minutes</th>
<th>10 minutes</th>
<th>15 minutes</th>
</tr>
</thead>
</table>

A

- DMSO control
  - DLM
  - TTM

- Deltamethrin 50 µg/ml (36.8 nI)
  - DLM
  - TTM

B

- CASPP 500 µg/ml (9.2 nI)
  - DLM
  - TTM

- CASPP 500 µg/ml (9.2 nI)
  - DLM
  - TTM
Figure 5.12  DLM and TTM EJP following frequency after injection of CASPP and deltamethrin

A. Injection of 9.2 nl of 500 µg/ml CASPP has a variable effect on DLM following frequency but does not affect TTM. B. Injection of 36.8 nl of 50 µg/ml deltamethrin caused loss of both DLM and TTM at 15 minutes after injection. DMSO vehicle had no effect during this time. Dots represent failed EJPs.

5.3 Discussion

The purpose of this chapter has been to evaluate the GFS as a tool to study insecticide mode of action. Due to time constraints and the elimination of some recordings because of quality, the sample size for some treatments is relatively low. Therefore, conclusions drawn in this chapter are speculative and will require further validation.

From the outset it was predicted that compounds with a target at the cholinergic synapse would be easily identified. This is because the PSI to DLMn synapse is cholinergic and the synapse between GF and TTMn is electrochemical (Fig. 5.1A), therefore compounds that target the cholinergic synapse would primarily affect the DLM and not the TTM. This was confirmed by CASPP, a compound that inhibits VACHT and reduces ACh release at the synapse (Sluder et al., 2012; chapter 1). CASPP caused an increase in DLM response latency and a decrease in DLM following frequency, suggestive of a delay in signal transmission and failure in the network. These results can be well explained by CASPP acting to reduce the availability of ACh-loaded vesicles. Targeting the cholinergic synapse was further verified by applying a stimulus direct to the thorax, bypassing the cholinergic synapse and returning DLM following frequency to control levels. However, at the highest concentration tested, CASPP also caused a modest reduction in following frequency at the TTM. This was not completely unexpected because the previous chapters have hinted a second, unknown, mode of action for CASPP (chapter 3.2.6/3.3.2). Compounds A and B were tested as potential inhibitors of VACHT. However, a clear distinction between the two compounds was found. Compound A had an effect on TTM following frequency, not consistent with a cholinergic target. Compound B affected only DLM, consistent with a target of the VACHT. An advantage of the GFS in this experiment has been to distinguish a second mode of action when the principle action is at the cholinergic synapse.
In chapter 3 I showed that when VACHT is overexpressed, the effect of CASPP is reduced. In this chapter, although cha>VACHT^{(+SYN)} flies showed increased resistance to CASPP, they displayed a similar effect with respect to CASPP to the DLM and an even more pronounced effect on response latency at the same concentrations as control. A reduction to TTM following frequency was also observed at concentrations lower than in controls. This was unexpected because larval recordings showed that cha>VACHT^{(+SYN)} larvae, after CASPP treatment were not different to untreated parental controls (chapter 3.2.4). However, an important difference between the larval recordings and GFS recordings is that larval recordings were carried out passively with no stimulation applied. GFS recordings were stimulated to trigger an EJP conducted by the GFS. Chapter 4 suggests that cha>VACHT^{(+SYN)} animals have an increased probability of SV release with no increase in SV number. If this is the case, then it is possible that each stimulus applied during GFS recordings may induce more SVs to fuse in cha>VACHT^{(+SYN)} than in control animals and the time required to recycle SVs may create a lag before the next EJP can be stimulated. It could be speculated that the increased effect to the TTM could be due to an unknown fitness cost caused by overexpressing the VACHT that makes the flies more vulnerable to this secondary effect of CASPP.

A second target at the cholinergic synapse tested was the nAChR. My results agree with the literature in suggesting that Dα7 is of high importance in the PSI to DLMn synapse. Flies null for Dα6 did not show any difference to control, suggesting that this subunit is not in the GFS pathway. This suggests that the GFS may not be a good model to screen compounds that target Dα6. It is possible that null Dα6 flies are able to substitute Dα6 with another, similar subunit such as Dα5 which is closely related to Dα6 (Sattelle et al., 2005). In wild-type flies the Dα6 subunit may be present at the PSI to DLMn synapse at low levels, explaining the effect of spinosad on increasing response latency. However, this result could also be explained if spinosad had low binding affinity to other nAChR subunits. Compound C, for which cross resistance to spinosad was found in null Dα6 flies (private communication, Syngenta), also increased response latency in the DLM. This supports a target at the cholinergic synapse but appears to be more potent than spinosad, potentially indicating a broader mode of action. Another observation made during these recordings was the unusual shape of the DLM EJPs. Four out of seven flies showed an unusually shaped DLM response, the TTM response was normal, suggesting this was due to an effect at the cholinergic synapse. There was also an effect on TTM following frequency at the highest dose tested. It is surprising that a compound
affects the TTM following frequency but not the DLM, because the pathway to the DLM is generally considered weaker due to the presence of the chemical synapse. This result may therefore be due to a relatively low sample size. I have shown that the GFS can distinguish a target at the cholinergic synapse. This technique would be of higher value if different targets within the cholinergic synapse were discernable. Preliminary observations indicate that compounds targeting the presynaptic release of ACh (CASPP, compound B) have a predominant effect on DLM following frequency. This could also be said of inhibition of the nAChR (as suggested by null Dα7 and MLA). However, an allosteric modulator of the nAChR could be more likely to have a stronger effect on response latency by affecting gating kinetics (spinosad, compound C). This is speculative and would require further experiments with a broader range of compounds.

The GFS can indicate a mode of action at the VGSC, demonstrated by effect of DCJW on DLM and TTM. However, unexpectedly DCJW only had this affect at the lowest concentration tested. DCJW causes pseudoparalysis, where insects appear paralysed but will twitch in response to a mechanical stimulus. Many VGSC blockers are believed to be voltage dependent, and are selective for VGSC in action potential initiation zones where the membrane potential is usually higher (Silver et al., 2010). Manduca sexta motoneurons, which were resistant to DCJW, could be made sensitive by depolarising with a high K⁺ solution (Wing et al., 1998). Therefore, it may be that at low concentrations of DCJW, where no paralysis occurs, all VGSC are vulnerable to block by DCJW. However, at high concentrations, VGSC at action potential initiation zones in the CNS are quickly inhibited, causing pseudoparalysis. This would mean that VGSC in axons and post-synaptic neurons further down neuronal pathways would remain in the resting state and so would not be vulnerable to block by DCJW. This theory could explain why higher concentrations of DCJW do not affect responses to stimulation of the GFS.

A drawback of the GFS is that it does not give good confirmation of target in compounds that have an excitatory effect. This was demonstrated by deltamethrin which showed no effect after feeding/contact application and a loss of EJPs after injection, and by fipronil, which only caused reduced following frequency in the TTM at the two lower concentrations applied. It was unexpected that a compound would affect the TTM but not DLM, a possible explanation is that the pathway to the TTM contains more GABA-gated chloride channels. Alternatively, the different effects of fipronil at different concentrations may be due to effects on glutamate-gated chloride channels (Zhao et al., 2004). These are known to be present at extra-synaptic
regions of insect somatic muscle (Wolstenholme, 2012). Another disadvantage may be the inability to distinguish between a compound that targets the NMJ and those that target the VGSC, because they affect both muscles, demonstrated by DCJW and philanthotoxin. However, this model is able to separate these modes of action from those at the cholinergic synapse.

5.4 Conclusions

The results presented in this chapter suggest that the GFS has potential to be used as a medium-throughput screen to identify insecticidal mode of action. However, my results are mixed and clearly further development work is required to achieve this goal. The GFS seems better suited for detecting a mode of action at the cholinergic synapse, which can be confirmed by thoracic stimulation. Application by feeding also allows compounds which require metabolism to their active component to be tested, this is especially valuable where the active metabolite is unknown. An indication of the effect of a compound can be achieved with a relatively low sample size. The drawbacks of this model are the inability to detect a mode of action that is excitatory and the risk that testing surviving flies from mortality assays selects those flies that are resistant to the compound. Overall, this technique would provide a valuable tool as part of an array of assays designed to determine mode of action but on its own it is limited.
<table>
<thead>
<tr>
<th>Treatment</th>
<th>DLM RL (ms)</th>
<th>P</th>
<th>DLM amplitude (mV)</th>
<th>P</th>
<th>DLM FF /10</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>CASPP 1 µg/ml</td>
<td>1.62 ± 0.05</td>
<td>0.2</td>
<td>53.6 ± 2.5</td>
<td>0.5</td>
<td>3.0 ± 0.5</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>CASPP 5 µg/ml</td>
<td>1.76 ± 0.07</td>
<td>0.004</td>
<td>52.4 ± 2.7</td>
<td>0.4</td>
<td>0.8 ± 0.2</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>CASPP 10 µg/ml</td>
<td>2.06 ± 0.1</td>
<td>&lt; 0.001</td>
<td>49.7 ± 4.3</td>
<td>0.07</td>
<td>1.0 ± 0.6</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Compound A 50 µg/ml</td>
<td>1.62 ± 0.08</td>
<td>0.07</td>
<td>50.9 ± 4.8</td>
<td>0.4</td>
<td>1.5 ± 0.2</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Compound A 100 µg/ml</td>
<td>1.65 ± 0.1</td>
<td>0.06</td>
<td>37.1 ± 8.9</td>
<td>0.002</td>
<td>1.2 ± 0.4</td>
<td>&lt; 0.001</td>
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<td>Compound A 300 µg/ml</td>
<td>1.56 ± 0.06</td>
<td>0.5</td>
<td>57.1 ± 4.4</td>
<td>1.0</td>
<td>0.8 ± 0.2</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Compound B 20 µg/ml</td>
<td>1.65 ± 0.03</td>
<td>0.2</td>
<td>53.2 ± 2.7</td>
<td>0.8</td>
<td>1.6 ± 0.5</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Compound B 40 µg/ml</td>
<td>1.60 ± 0.03</td>
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<td>37.4 ± 2.5</td>
<td>0.004</td>
<td>1.3 ± 0.5</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Compound B 100 µg/ml</td>
<td>1.72 ± 0.2</td>
<td>0.006</td>
<td>53.7 ± 4.4</td>
<td>0.8</td>
<td>0.9 ± 0.2</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Cha&gt;VACHT&lt;sup&gt;(SYN)&lt;/sup&gt; control</td>
<td>1.47 ± 0.01</td>
<td>59.5 ± 1.3</td>
<td>7.1 ± 2.0</td>
<td>0.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cha&gt;VACHT&lt;sup&gt;(SYN)&lt;/sup&gt; + CASPP 1 µg/ml</td>
<td>1.44 ± 0.03</td>
<td>1.0</td>
<td>57.6 ± 4.6</td>
<td>1.0</td>
<td>6.0 ± 0.8</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Cha&gt;VACHT&lt;sup&gt;(SYN)&lt;/sup&gt; + CASPP 5 µg/ml</td>
<td>1.52 ± 0.08</td>
<td>1.0</td>
<td>55.7 ± 3.7</td>
<td>1.0</td>
<td>3.7 ± 0.9</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Cha&gt;VACHT&lt;sup&gt;(SYN)&lt;/sup&gt; + CASPP 10 µg/ml</td>
<td>2.32 ± 0.6</td>
<td>&lt; 0.001</td>
<td>58.2 ± 5.4</td>
<td>1.0</td>
<td>0.4 ± 0.2</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Treatment</th>
<th>TTM RL (ms)</th>
<th>P</th>
<th>TTM amplitude (mV)</th>
<th>P</th>
<th>TTM FF /10</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>CASPP 1 µg/ml</td>
<td>1.1 ± 0.06</td>
<td>0.7</td>
<td>19.5 ± 0.9</td>
<td>0.7</td>
<td>7.6 ± 1.0</td>
<td>0.1</td>
</tr>
<tr>
<td>CASPP 5 µg/ml</td>
<td>1.1 ± 0.04</td>
<td>1.0</td>
<td>21.0 ± 2.2</td>
<td>1.0</td>
<td>8.3 ± 1.0</td>
<td>0.5</td>
</tr>
<tr>
<td>CASPP 10 µg/ml</td>
<td>1.2 ± 0.1</td>
<td>0.1</td>
<td>21.5 ± 1.8</td>
<td>1.0</td>
<td>7.2 ± 1.1</td>
<td>0.01</td>
</tr>
<tr>
<td>Compound A 50 µg/ml</td>
<td>1.0 ± 0.01</td>
<td>0.9</td>
<td>16.8 ± 2.0</td>
<td>0.4</td>
<td>6.2 ± 1.7</td>
<td>0.01</td>
</tr>
<tr>
<td>Compound A 100 µg/ml</td>
<td>1.02 ± 0.05</td>
<td>1.0</td>
<td>19.8 ± 4.2</td>
<td>0.9</td>
<td>7.8 ± 2.3</td>
<td>0.5</td>
</tr>
<tr>
<td>Compound A 300 µg/ml</td>
<td>1.05 ± 0.06</td>
<td>1.0</td>
<td>19.0 ± 3.2</td>
<td>0.8</td>
<td>6.0 ± 1.9</td>
<td>0.003</td>
</tr>
<tr>
<td>Compound B 20 µg/ml</td>
<td>1.1 ± 0.1</td>
<td>0.7</td>
<td>19.0 ± 3.7</td>
<td>0.9</td>
<td>7.9 ± 1.9</td>
<td>0.1</td>
</tr>
<tr>
<td>Compound B 40 µg/ml</td>
<td>1.03 ± 0.06</td>
<td>1.0</td>
<td>17.5 ± 3.2</td>
<td>0.7</td>
<td>10.0 ± 0.0</td>
<td>0.8</td>
</tr>
<tr>
<td>Compound B 100 µg/ml</td>
<td>1.2 ± 0.01</td>
<td>0.4</td>
<td>26.6 ± 2.2</td>
<td>0.6</td>
<td>10.0 ± 0.05</td>
<td>0.8</td>
</tr>
<tr>
<td>Cha&gt;VACHT&lt;sup&gt;(SYN)&lt;/sup&gt; control</td>
<td>1.04 ± 0.01</td>
<td>21.8 ± 0.8</td>
<td>9.4 ± 0.1</td>
<td>1.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cha&gt;VACHT&lt;sup&gt;(SYN)&lt;/sup&gt;</td>
<td>1.03 ± 0.03</td>
<td>1.0</td>
<td>21.2 ± 1.8</td>
<td>1.0</td>
<td>9.0 ± 0.7</td>
<td>1.0</td>
</tr>
<tr>
<td>Cha&gt;VACHT&lt;sup&gt;(SYN)&lt;/sup&gt; + CASPP 1 µg/ml</td>
<td>1.09 ± 0.07</td>
<td>1.0</td>
<td>19.8 ± 1.6</td>
<td>1.0</td>
<td>6.4 ± 1.1</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Cha&gt;VACHT&lt;sup&gt;(SYN)&lt;/sup&gt; + CASPP 5 µg/ml</td>
<td>1.06 ± 0.08</td>
<td>1.0</td>
<td>16.6 ± 1.6</td>
<td>1.0</td>
<td>5.7 ± 1.8</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Cha&gt;VACHT&lt;sup&gt;(SYN)&lt;/sup&gt; + CASPP 10 µg/ml</td>
<td>1.07 ± 0.7</td>
<td>1.0</td>
<td>16.1 ± 2.7</td>
<td>0.6</td>
<td>6.6 ± 1.7</td>
<td>0.006</td>
</tr>
</tbody>
</table>
Table 5.7  Mean values of response latency, amplitude and following frequency in DLM and TTM muscles after compound treatment targeted at nAChR. Values are mean ± SEM

<table>
<thead>
<tr>
<th>Treatment</th>
<th>DLM RL (ms)</th>
<th>P</th>
<th>DLM amplitude (mV)</th>
<th>P</th>
<th>DLM FF /10</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Null Da7</td>
<td>5.17 ± 0.4</td>
<td>&lt; 0.001</td>
<td>53.1 ± 3.2</td>
<td>0.7</td>
<td>0.4 ± 0.2</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Null Da6</td>
<td>1.37 ± 0.1</td>
<td>0.4</td>
<td>58.0 ± 3.2</td>
<td>1.0</td>
<td>6.1 ± 0.8</td>
<td>0.4</td>
</tr>
<tr>
<td>Spinosad 8 µg/ml</td>
<td>1.50 ± 0.06</td>
<td>1.0</td>
<td>67.9 ± 2.8</td>
<td>0.4</td>
<td>6.8 ± 0.8</td>
<td>1.0</td>
</tr>
<tr>
<td>Spinosad 16 µg/ml</td>
<td>1.40 ± 0.08</td>
<td>0.7</td>
<td>48.0 ± 7.8</td>
<td>0.2</td>
<td>6.5 ± 0.8</td>
<td>0.9</td>
</tr>
<tr>
<td>Spinosad 32 µg/ml</td>
<td>1.66 ± 0.1</td>
<td>0.02</td>
<td>56.9 ± 5.0</td>
<td>1.0</td>
<td>7.0 ± 0.8</td>
<td>1.0</td>
</tr>
<tr>
<td>Compound C 5 µg/ml</td>
<td>1.77 ± 0.2</td>
<td>&lt; 0.001</td>
<td>55.6 ± 4.4</td>
<td>0.9</td>
<td>6.6 ± 1.0</td>
<td>0.9</td>
</tr>
<tr>
<td>Compound C 10 µg/ml</td>
<td>1.68 ± 0.05</td>
<td>0.05</td>
<td>54.8 ± 6.4</td>
<td>0.9</td>
<td>8.2 ± 0.4</td>
<td>0.6</td>
</tr>
<tr>
<td>Compound C 50 µg/ml</td>
<td>1.76 ± 0.1</td>
<td>&lt; 0.001</td>
<td>56.1 ± 8.5</td>
<td>0.9</td>
<td>8.0 ± 0.5</td>
<td>0.8</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Treatment</th>
<th>TTM RL (ms)</th>
<th>P</th>
<th>TTM amplitude (mV)</th>
<th>P</th>
<th>TTM FF /10</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Null Da7</td>
<td>1.1 ± 0.05</td>
<td>0.5</td>
<td>17.7 ± 2.9</td>
<td>0.3</td>
<td>8.9 ± 0.7</td>
<td>0.6</td>
</tr>
<tr>
<td>Null Da6</td>
<td>1.07 ± 0.04</td>
<td>0.8</td>
<td>22.0 ± 4.1</td>
<td>1.0</td>
<td>8.5 ± 0.7</td>
<td>0.2</td>
</tr>
<tr>
<td>Spinosad 8 µg/ml</td>
<td>1.06 ± 0.1</td>
<td>1.0</td>
<td>28.1 ± 2.1</td>
<td>0.3</td>
<td>9.9 ± 0.1</td>
<td>0.7</td>
</tr>
<tr>
<td>Spinosad 16 µg/ml</td>
<td>1.04 ± 0.05</td>
<td>1.0</td>
<td>19.2 ± 1.6</td>
<td>0.8</td>
<td>9.4 ± 0.6</td>
<td>1.0</td>
</tr>
<tr>
<td>Spinosad 32 µg/ml</td>
<td>1.09 ± 0.09</td>
<td>0.9</td>
<td>19.0 ± 3.8</td>
<td>0.8</td>
<td>9.3 ± 0.5</td>
<td>1.0</td>
</tr>
<tr>
<td>Compound C 5 µg/ml</td>
<td>1.19 ± 0.1</td>
<td>0.08</td>
<td>27.3 ± 3.1</td>
<td>0.4</td>
<td>8.5 ± 1.1</td>
<td>0.5</td>
</tr>
<tr>
<td>Compound C 10 µg/ml</td>
<td>1.10 ± 0.04</td>
<td>0.8</td>
<td>27.7 ± 2.7</td>
<td>0.3</td>
<td>9.4 ± 0.4</td>
<td>1.0</td>
</tr>
<tr>
<td>Compound C 50 µg/ml</td>
<td>1.17 ± 0.05</td>
<td>0.2</td>
<td>21.6 ± 4.0</td>
<td>1.0</td>
<td>6.9 ± 1.3</td>
<td>0.001</td>
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</table>
Table 5.8  Mean values of response latency, amplitude and following frequency in DLM and TTM muscles after compound treatment targeted at the VGSC. Values are mean ± SEM

<table>
<thead>
<tr>
<th>Treatment</th>
<th>DLM RL (ms)</th>
<th>P</th>
<th>DLM amplitude (mV)</th>
<th>P</th>
<th>DLM FF /10</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indoxacarb 1000 µg/ml</td>
<td>1.43 ± 0.04</td>
<td>0.3</td>
<td>57.5 ± 4.2</td>
<td>0.7</td>
<td>5.2 ± 1.1</td>
<td>0.03</td>
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<td>DCJW 0.5 µg/ml</td>
<td>1.79 ± 0.2</td>
<td>0.002</td>
<td>53.8 ± 5.1</td>
<td>0.7</td>
<td>4.2 ± 1.0</td>
<td>0.007</td>
</tr>
<tr>
<td>DCJW 2 µg/ml</td>
<td>1.49 ± 0.06</td>
<td>1.0</td>
<td>57.6 ± 3.4</td>
<td>1.0</td>
<td>7.6 ± 1.1</td>
<td>1.0</td>
</tr>
<tr>
<td>DCJW 4 µg/ml</td>
<td>1.46 ± 0.03</td>
<td>1.0</td>
<td>62.0 ± 4.3</td>
<td>0.9</td>
<td>7.5 ± 0.5</td>
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<td>60.6 ± 1.2</td>
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<td>7.6 ± 0.8</td>
<td>1.0</td>
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<tr>
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<td>1.0</td>
</tr>
<tr>
<td>Deltamethrin 0.5 µg/ml</td>
<td>1.47 ± 0.05</td>
<td>1.0</td>
<td>54.1 ± 6.7</td>
<td>0.8</td>
<td>6.6 ± 0.7</td>
<td>0.9</td>
</tr>
<tr>
<td>Compound D 4000 µg/ml</td>
<td>1.49 ± 0.08</td>
<td>0.8</td>
<td>54.0 ± 3.5</td>
<td>0.3</td>
<td>4.1 ± 1.3</td>
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</table>

<table>
<thead>
<tr>
<th>Treatment</th>
<th>TTM RL (ms)</th>
<th>P</th>
<th>TTM amplitude (mV)</th>
<th>P</th>
<th>TTM FF /10</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indoxacarb 1000 µg/ml</td>
<td>1.05 ± 0.04</td>
<td>1.0</td>
<td>22.3 ± 3.6</td>
<td>1.0</td>
<td>9.3 ± 0.3</td>
<td>0.8</td>
</tr>
<tr>
<td>DCJW 0.5 µg/ml</td>
<td>1.27 ± 0.1</td>
<td>0.002</td>
<td>27.5 ± 5.0</td>
<td>0.4</td>
<td>5.1 ± 1.2</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>DCJW 2 µg/ml</td>
<td>1.07 ± 0.05</td>
<td>1.0</td>
<td>26.0 ± 3.1</td>
<td>0.7</td>
<td>8.9 ± 0.8</td>
<td>0.9</td>
</tr>
<tr>
<td>DCJW 4 µg/ml</td>
<td>1.05 ± 0.03</td>
<td>1.0</td>
<td>21.8 ± 2.3</td>
<td>1.0</td>
<td>8.4 ± 0.4</td>
<td>0.3</td>
</tr>
<tr>
<td>Deltamethrin 0.01 µg/ml</td>
<td>1.18 ± 0.1</td>
<td>0.4</td>
<td>15.2 ± 3.0</td>
<td>0.2</td>
<td>8.6 ± 1.0</td>
<td>0.6</td>
</tr>
<tr>
<td>Deltamethrin 0.05 µg/ml</td>
<td>1.22 ± 0.2</td>
<td>0.1</td>
<td>17.6 ± 2.8</td>
<td>0.5</td>
<td>8.1 ± 1.0</td>
<td>0.1</td>
</tr>
<tr>
<td>Deltamethrin 0.5 µg/ml</td>
<td>1.04 ± 0.03</td>
<td>1.0</td>
<td>18.2 ± 1.6</td>
<td>0.6</td>
<td>9.0 ± 0.5</td>
<td>0.9</td>
</tr>
<tr>
<td>Compound D 4000 µg/ml</td>
<td>1.03 ± 0.08</td>
<td>0.8</td>
<td>19.0 ± 2.8</td>
<td>0.4</td>
<td>9.8 ± 0.1</td>
<td>0.4</td>
</tr>
</tbody>
</table>
Table 5.9  Mean values of response latency, amplitude and following frequency in DLM and TTM muscles after compound treatment targeted at the NMJ. Values are mean ± SEM

<table>
<thead>
<tr>
<th>Treatment</th>
<th>DLM RL (ms)</th>
<th>P</th>
<th>DLM amplitude (mV)</th>
<th>P</th>
<th>DLM FF /10</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Philanthotoxin</td>
<td>1.65 ± 0.06</td>
<td>&lt; 0.001</td>
<td>52.1 ± 4.9</td>
<td>0.2</td>
<td>8.4 ± 0.5</td>
<td>0.1</td>
</tr>
<tr>
<td>3000 µg/ml</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Compound E 100</td>
<td>1.51 ± 0.08</td>
<td>0.9</td>
<td>50.8 ± 4.3</td>
<td>0.4</td>
<td>5.1 ± 0.7</td>
<td>0.1</td>
</tr>
<tr>
<td>µg/ml</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Compound E 500</td>
<td>1.46 ± 0.04</td>
<td>1.0</td>
<td>51.1 ± 3.7</td>
<td>0.4</td>
<td>5.5 ± 0.8</td>
<td>0.2</td>
</tr>
<tr>
<td>µg/ml</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Compound E 1000</td>
<td>1.46 ± 0.03</td>
<td>1.0</td>
<td>52.7 ± 4.8</td>
<td>0.5</td>
<td>3.5 ± 0.9</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>µg/ml</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Treatment</th>
<th>TTM RL (ms)</th>
<th>P</th>
<th>TTM amplitude (mV)</th>
<th>P</th>
<th>TTM FF /10</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Philanthotoxin</td>
<td>1.14 ± 0.04</td>
<td>0.03</td>
<td>22.6 ± 3.0</td>
<td>0.8</td>
<td>9.3 ± 0.6</td>
<td>0.8</td>
</tr>
<tr>
<td>3000 µg/ml</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Compound E 100</td>
<td>1.04 ± 0.07</td>
<td>1.0</td>
<td>21.1 ± 2.4</td>
<td>1.0</td>
<td>8.1 ± 0.5</td>
<td>0.3</td>
</tr>
<tr>
<td>µg/ml</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Compound E 500</td>
<td>1.12 ± 0.04</td>
<td>0.5</td>
<td>22.0 ± 3.1</td>
<td>1.0</td>
<td>7.7 ± 1.2</td>
<td>0.06</td>
</tr>
<tr>
<td>µg/ml</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Compound E 1000</td>
<td>1.07 ± 0.06</td>
<td>1.0</td>
<td>19.3 ± 2.5</td>
<td>0.8</td>
<td>7.6 ± 1.5</td>
<td>0.08</td>
</tr>
<tr>
<td>µg/ml</td>
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<td></td>
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</table>

Table 5.10  Mean values of response latency, amplitude and following frequency in DLM and TTM muscles after compound treatment targeted at the GABA-gated chloride channel. Values are mean ± SEM

<table>
<thead>
<tr>
<th>Treatment</th>
<th>DLM RL (ms)</th>
<th>P</th>
<th>DLM amplitude (mV)</th>
<th>P</th>
<th>DLM FF /10</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fipronil 2 µg/ml</td>
<td>1.36 ± 0.06</td>
<td>0.2</td>
<td>47.9 ± 6.6</td>
<td>0.2</td>
<td>6.7 ± 1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>µg/ml</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fipronil 8 µg/ml</td>
<td>1.43 ± 0.04</td>
<td>0.9</td>
<td>57.7 ± 4.0</td>
<td>1.0</td>
<td>5.5 ± 1.4</td>
<td>0.3</td>
</tr>
<tr>
<td>µg/ml</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fipronil 16 µg/ml</td>
<td>1.54 ± 0.04</td>
<td>0.6</td>
<td>53.8 ± 7.2</td>
<td>0.8</td>
<td>5.9 ± 1.0</td>
<td>0.6</td>
</tr>
<tr>
<td>µg/ml</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Treatment</th>
<th>TTM RL (ms)</th>
<th>P</th>
<th>TTM amplitude (mV)</th>
<th>P</th>
<th>TTM FF /10</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fipronil 2 µg/ml</td>
<td>0.94 ± 0.09</td>
<td>0.3</td>
<td>25.1 ± 3.4</td>
<td>0.3</td>
<td>7.0 ± 1.7</td>
<td>0.02</td>
</tr>
<tr>
<td>µg/ml</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fipronil 8 µg/ml</td>
<td>1.0 ± 0.03</td>
<td>0.8</td>
<td>19.8 ± 4.4</td>
<td>0.2</td>
<td>6.7 ± 1.7</td>
<td>0.007</td>
</tr>
<tr>
<td>µg/ml</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fipronil 16 µg/ml</td>
<td>1.15 ± 0.08</td>
<td>0.3</td>
<td>20.6 ± 2.6</td>
<td>0.07</td>
<td>8.0 ± 0.6</td>
<td>0.3</td>
</tr>
<tr>
<td>µg/ml</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</table>
Table 5.11  Mean values of response latency, amplitude and following frequency in DLM and TTM during head and thoracic stimulation. Values are mean ± SEM

<table>
<thead>
<tr>
<th>Treatment</th>
<th>DLM RL (ms)</th>
<th>P</th>
<th>DLM amplitude (mV)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control CNS</td>
<td>1.47 ± 0.01</td>
<td></td>
<td>7.2 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>Control thoracic</td>
<td>0.76 ± 0.09</td>
<td></td>
<td>9.0 ± 1.0</td>
<td></td>
</tr>
<tr>
<td>Null Da7 CNS</td>
<td>5.17 ± 0.4</td>
<td>&lt; 0.001</td>
<td>0.4 ± 0.2</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Null Da7 thoracic</td>
<td>0.77 ± 0.1</td>
<td>1.0</td>
<td>8.1 ± 1.6</td>
<td>1.0</td>
</tr>
<tr>
<td>CASPP 10 µg/ml CNS</td>
<td>2.06 ± 0.1</td>
<td>&lt; 0.001</td>
<td>1.0 ± 0.6</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>CASPP 10 µg/ml thoracic</td>
<td>0.88 ± 0.02</td>
<td>0.04</td>
<td>8.5 ± 1.4</td>
<td>1.0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Treatment</th>
<th>TTM RL (ms)</th>
<th>P</th>
<th>TTM amplitude (mV)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control CNS</td>
<td>1.05 ± 0.01</td>
<td></td>
<td>9.4 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>Control thoracic</td>
<td>0.62 ± 0.06</td>
<td></td>
<td>10.0 ± 0.0</td>
<td></td>
</tr>
<tr>
<td>Null Da7 CNS</td>
<td>1.1 ± 0.05</td>
<td>0.5</td>
<td>8.9 ± 0.7</td>
<td>0.6</td>
</tr>
<tr>
<td>Null Da7 thoracic</td>
<td>0.78 ± 0.02</td>
<td>0.2</td>
<td>10.0 ± 0.0</td>
<td>1.0</td>
</tr>
<tr>
<td>CASPP 10 µg/ml CNS</td>
<td>1.2 ± 0.1</td>
<td>0.1</td>
<td>7.2 ± 1.1</td>
<td>0.01</td>
</tr>
<tr>
<td>CASPP 10 µg/ml thoracic</td>
<td>0.62 ± 0.07</td>
<td>1.0</td>
<td>9.7 ± 0.3</td>
<td>0.6</td>
</tr>
</tbody>
</table>
Chapter 6

General Discussion

6.1 Conclusions

Development of insecticidal compounds requires elucidation of mode of action. In an ideal scenario a well characterised system would give an output specific to different modes of action. Unfortunately in vivo models are rarely simple and many insecticidal compounds have secondary modes of action. For example, fipronil is classified as a blocker of GABA-gated chloride channels and knockdown of a GABA receptor RDL gene in the small brown planthopper reduced susceptibility to this compound (Wei et al., 2015). However, there is also evidence that fipronil acts on the glutamate-gated chloride channels and glycine receptors (Zhao et al., 2005; Islam and Lynch, 2012). Expense and time required for screening are also factors in considering a mode of action screen. I aimed to assess Drosophila as an experimental model for studying insecticide mode of action. Drosophila as a model offers a low cost, low maintenance organism with a relatively simple CNS. In larvae I confirmed that the spiroindoline class of insecticides has a mode of action at the VACHT and determined the effect on ACh release at synapses. Larval tracking showed that spiroindoline compound 5Cl-CASPP (CASPP) caused decreased speed and distance travelled and mortality assays showed that larvae that overexpressed the wild-type or a resistant form of VACHT were less sensitive to CASPP.

Electrophysiological recordings from motoneurons that receive cholinergic input showed that CASPP decreased quantal release frequency but not amplitude. No change to postsynaptic response to ACh confirmed that this was a presynaptic effect. This effect was rescued in transgenic larvae overexpressing either wild-type or a resistant form of VACHT. This demonstrates that Drosophila larvae can be successfully used to confirm mode of action and the value of the genetic tools in facilitating this. However, the electrophysiological assays which ultimately confirmed the effect of CASPP on ACh release at the synapse do not provide a fast screening method. Using adult Drosophila I aimed to develop a medium through-put mode of
action screen that utilises the GFS escape response network. This system proved to be poor in identifying excitatory modes of action and even classification of inhibitory compounds was limited. The neurons involved in the network and synapse types are known (Balgburn et al., 1999; Allen et al., 2006; Fayyazuddin et al., 2006; Allen and Murphey, 2007; Phelan et al., 2008) but there is still a lot about this system that is uncharacterised. For example, the TTMn and DLMn have other unidentified inputs, one of which can be triggered by a looming stimuli (Fotowat et al., 2009). It is not known how these inputs may interfere with recordings from TTM and DLM. This makes predicting the output after insecticide treatment difficult because other neuronal networks that synapse with neurons involved in the GFS may influence output to the muscles. This system did show potential in indicating cholinergic mode of action because a mode of action here caused characteristic loss of EJP in the DLM but not the TTM. However, some compounds with this proposed mode of action (e.g. CASPP and compound A) affected both muscles and it was not known if this was because of a secondary effect of the compound or an unknown interaction within the GFS. Compound application by feeding/contact had the benefit of being able to test compounds that are metabolised to their active component but also presented the problem that testing surviving flies may be selecting those which were resistant. Injection of compound does not allow time for resistance to form and so may be the better method of compound application, however some variability was also found with this method and excitatory compounds had either an inhibitory effect or no effect. I conclude that the GFS as a model for studying insecticide mode of action requires a great deal more characterisation of how changes within the system affect output. In this study, greater success was had using larval Drosophila, although more time consuming, confirmation of target was more reliably achieved. However, further experiments using compounds with other mode of actions would be required to determine the true value of this model. This project also highlights the value of a screening model using Drosophila because of the ease of genetic manipulation and new tools are frequently developed. For example, CRISPR/cas technology that allows gene editing is now available in Drosophila (Bassett and Liu, 2014). This new technique has the added benefit that altered genes will be expressed at endogenous levels, something that cannot be controlled when using the GAL4-UAS mode of gene expression.

Cholinergic tone has been linked to many neuronal diseases including Alzheimer’s, schizophrenia and epilepsy (Auld et al., 2002; Terry and Bucafeusco, 2003; Dani and Bertrand, 2007; Schliebs and Arendt, 2011; Hall et al., 2014). Many models for
studying cholinergic synapses are at the NMJ of, for example, mouse, rat and frog. Central cholinergic neurons are not easily accessible in vivo in these animals and consistent targeting of the same neuron impossible. Central cholinergic synapses have roles in cognitive function whereas those at the NMJ predominantly control muscle contraction. These differing roles of the cholinergic synapse may mean regulation of ACh release is controlled differently at central synapses compared to peripheral ones. Drosophila larvae have easily accessible central cholinergic synapses to well characterised motoneurons (aCC and RP2) that can be repeatedly identified. Levels of VACHT have been found to change in Alzheimer’s and Huntington’s and the VACHT is also involved in the immune response (Chen et al., 2011; Efange et al., 1997; Smith et al., 2006; Ribeiro et al., 2012). Using Drosophila I have shown that inhibition of the VACHT leads to decreased ACh release at the cholinergic synapse and overexpression of VACHT leads to increased ACh release. Therefore VACHT expression is able to govern ACh release and may present a new therapeutic target in disease. This could be achieved by targeting a signalling pathway that regulates VACHT expression. For example, when neuronal cell lines were transfected with transcription factor Lhx8 cDNA, expression of ChAT and VACHT increased and ACh released by the cells also increased (Li et al., 2014). Drugs could also be targeted at the VACHT itself. A resistant form of VACHT with a point mutation (Y49N) showed increased ACh release after treatment with CASPP. It is possible that this is due to an interaction between CASPP and the VACHT and suggests that VACHT function could be influenced by drugs. The central cholinergic synapses in Drosophila could provide a model to test drugs targeted at VACHT. Increasing understanding of these synapses will further increase the value of this model. This thesis has contributed to characterisation of the cholinergic synapse to the aCC motoneuron by determining that the most prevalent subunits of the nAChR in aCC motoneurons are Dα2, Dα5 and Dα6. In addition to contributing to information known about this synapse, this knowledge might contribute to future work in understanding the function of subunits of the Drosophila nAChR.

There are various estimates of the number of vesicular transporters per vesicle, from 1 – 3 to around 10 copies per vesicle at central synapses (Van der Kloot, 2003; Takamori et al., 2006). At the Drosophila NMJ VGLUT mutants caused reduced frequency but not amplitude of miniature EJPs (quantal release) with no change to postsynaptic response to glutamate (Daniels et al., 2006). The authors concluded that there is one VGLUT per SV and that this is sufficient to fill a SV to completion. My results using CASPP suggest that the same might be true at Drosophila central
pre-motoneuron cholinergic synapses. After CASPP treatment mini frequency was reduced but amplitude remained the same and postsynaptic response to ACh did not appear to be changed. If there were multiple VACHE per SV it might be expected that some SVs would have fewer functional VACHE and so ACh load would be reduced. However, here it would seem that SVs are either loaded or empty, consistent with one transporter per vesicle that is either functional or blocked (Fig. 6.1). Decreased frequency but not amplitude is in agreement with studies in the literature at *Drosophila* (VGLUT) and snake (VACHE) NMJ (Parsons *et al.*, 1999; Daniels *et al.*, 2006). However studies at mouse, frog and rat NMJ (VACHE) and using isolated SVs from rat cerebral cortex and hippocampus (VGLUT) also reported decreased transmitter load (Searl *et al.*, 1999; van der Kloot, 2000; Wilson *et al.*, 2005; Prado *et al.*, 2006; Lima *et al.*, 2010 and Rodriguez *et al.*, 2013). This suggests that number of transporters per vesicle varies between species.

![Figure 6.1](image)

**Figure 6.1** Model of single transporter per SV vs. multiple transporters.  
**A.** If SVs had only one VACHE then after CASPP treatment this VACHE would be either functional or blocked, leading to either loaded or empty vesicles. **B.** If SVs contained multiple VACHE then after CASPP treatment some but not all VACHE per SV may be blocked, leading to decreased ACh content.
There is ongoing discussion about how quantal size is regulated. There are two proposed methods of vesicle filling: the ‘steady state’ model and the ‘set point’ model (Williams et al., 1997). Steady state filling assumes a non-specific leak (i.e. not through the transporter) of neurotransmitter out of the SV that reaches an equilibrium with transport of neurotransmitter into the SV. Overexpression of the transporter would lead to increased quantal size because leak does not increase enough to counteract increased input, and so total content is increased (Fig. 6.2A). This appears to be the case at the NMJ of *Xenopus*, *Drosophila* and in cultured hippocampal neurons from rat (Song et al., 1997; Daniels et al., 2004; Wilson et al., 2005) where overexpression of the transporter caused increased amplitude of spontaneous quantal release. The set point model assumes there is a set point that vesicles are filled to, achieved through a feedback mechanism or if a component of the neurotransmitter production/packaging machinery is limiting. In this model increased transporter would lead to increased speed of loading but not increased total content (Fig. 6.2B). The central cholinergic synapses in *Drosophila* appear to abide by this set point model because increased transporter did not lead to increased quantal size.

**Figure 6.2** Steady state and set point models of SV loading.

A. In the steady state model ACh leak increases with ACh load. When VACHT is increased inflow exceeds outflow and ACh load is increased. B. In the set point model ACh load is determined by a feedback mechanism or a limiting factor in the ACh cycle. When VACHT is increased, speed of filling increases but ACh load does not increase.
Interestingly, when a VACHT variant that lacks a glutamine from a 13 glutamine polyQ domain (VACHT\textsuperscript{−(SYN)}\textsuperscript{−}) is overexpressed, minis increased in amplitude and frequency. Normal length polyQ domains are proposed to have roles in protein-protein interactions, transcriptional regulation, RNA binding and signalling (Schaefer et al., 2012). If the polyQ domain is involved in the feedback mechanism that sets the point of filling, the variant with a missing glutamine may have altered this feedback mechanism causing ACh load to be increased. Alternatively if steady state filling applied and the -Q variant of the VACHT is more successfully localised to SV membrane, the increase in VACHT per vesicle may be sufficient to influence detectable ACh load. The polyQ domain of the VACHT is not found in mammals and may therefore present a unique target for insecticides.

Overexpression of the VACHT variants with 12 or 13 glutamines in the polyQ domain both increased frequency of minis. This does not appear to be due to increased number of SVs and so proposes a second role for VACHT in influencing probability of release. Such a role has been previously suggested in \textit{C.elegans} (Sandoval et al., 2006). In conclusion decreased active VACHT leads to decreased ACh release that could explain decreased cholinergic activity in neuronal diseases where a decrease in VACHT is reported. Overexpression of the VACHT leads to increased release of ACh and may suggest a novel therapeutic target in neuronal disease.

Overall this thesis has demonstrated that \textit{Drosophila} larvae provide a reliable low throughput model to identify insecticide mode of action and that the GFS in the adult has potential to identify compounds with a cholinergic target but requires further characterisation. This project also found that VACHT is able to govern ACh release at central pre-motoneuron cholinergic synapses in \textit{Drosophila} and puts forward \textit{Drosophila} as a model to study central cholinergic synapses \textit{in vivo}.

### 6.2 Outlook

Some areas of this project require further investigation to fully understand the mechanisms behind how the VACHT influences ACh release and to further test \textit{Drosophila} as a model to understand insecticide mode of action.

I have shown that the polyQ domain of VACHT may influence ACh loading. This overexpression of the missing Q variant also caused increased duration of SRCs. It
is not known if this is also the case when VACHT with the full 13 glutamine polyQ domain is overexpressed, therefore it would be beneficial for these experiments to be carried out because it may demonstrate that the polyQ domain also affects time-course of SV release in evoked events. It would also be important to determine how VACHT with different numbers of glutamines in the polyQ domain affects minis and SRCs. This could carried out using the GAL4-UAS system to overexpress different polyQ variants or using gene editing via the CRISPR/cas system. Gene editing has the advantage that altered proteins are expressed at endogenous levels, something that is not true of GAL4 driven expression.

I found that when a CASPP resistant form of VACHT was overexpressed mini frequency was increased after CASPP treatment. It is not known if this is because of an interaction between the wild-type and Y49N variants of VACHT or an interaction between CASPP and VACHTY49N. To try and distinguish between these two possibilities VACHTY49N would need to be expressed without the endogenous VACHT. This could be achieved by overexpressing the VACHTY49N in a null VACHT background or by gene editing of the endogenous VACHT using CRISPR/cas. This would also allow for observation of any fitness cost caused by this mutation that may be of importance for understanding resistance in the field.

In this project I found that the predominant subunits of nAChR expressed in the aCC motoneurons are Dα2, Dα5 and Dα6. It will be important to determine if altered VACHT function and expression altered subunit composition. Although response to ACh did not appear to be affected, characterisation of subunits would give greater confirmation of this result, or may indeed contradict it. This could be done using the same method of FACS sorting GFP labelled aCC motoneurons and quantification by RT-qPCR.

The GFS was used to test Drosophila as a model to study insecticide mode of action. Multiple mode of action of compounds and complexity of the GFS combined to produce inconsistent results. Further methods of compound application need to be tested and optimised. An ideal system would allow perfusion of the haemolymph to allow compounds to be washed out and tested at different concentrations in the same fly. This model could be further improved if excitatory compounds could be detected. This may be achieved by observing spontaneous activity (i.e. no applied stimulus) which would be expected to be increased under excitatory compound treatment.
References


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