GENETIC EVIDENCE FOR DIFFERENTIAL ACTIVITIES OF
Goα ISOFORMS IN DROSOPHILA MELANOGASTER

by

Vanessa Di Gioacchino

A thesis submitted to the Department of Biology
in conformity with the requirements for
the degree of Master of Science

Queen’s University
Kingston, Ontario, Canada
December 2014

Copyright © Vanessa Di Gioacchino, 2014
ABSTRACT

Mental illness is prevalent in our population and has been studied for decades but is still poorly understood. Understanding the genetic and biological mechanisms underlying these disorders can lead to significant improvements in diagnosis, treatment, and quality of life for patients suffering from mental disorders.

\( \text{Go} \alpha 47A \) is a candidate gene in a putative haloperidol-affected pathway that is highly expressed in the nervous system. The gene encodes nine transcripts and two isoforms, which are 98% identical and differ only in the N-terminal region. However, the individual roles for each of the two different proteins have not yet been discerned. Transgenic strains of \textit{Drosophila melanogaster} were generated using cDNA constructs corresponding to each of the two open reading frames (ORFs) under the control of UAS promoters. Expression of these transgenes was driven by a GAL4 driver with a similar spatial expression pattern to that of \( \text{Go} \alpha 47A \). These constructs were intended to be used in a genetic rescue in order to test the hypothesis that a mutant allele of \( \text{Go} \alpha 47A \) causes resistance to the antipsychotic haloperidol.

In the course of generating strains needed for the rescue, it was found that the expression of either ORF causes a crumpled wing phenotype and lethality, both with variable penetrance. This variability is likely due to expression level, since GAL80 can reduce the frequency of these phenotypes. Additionally, there is an interaction between the ORFs and the TM3 balancer, which could indicate an interaction between Go\( \alpha \) and a gene that is affected by the chromosomal rearrangements associated with the TM3 balancer. The majority of VD1 strains display crumpled wings and the expression of ORF1 can cause lethality. In contrast, two copies of the ORF2 transgene are required to cause crumpled wings. The difference in activities between the two ORFs is likely due to the few changes in the N-terminal region of the protein, which is
presumably involved in the interaction with the β and γ subunits. This finding suggests that small differences in a G-protein are sufficient to alter protein-protein interactions and subsequent signalling.
CO-AUTHORSHIP

In Chapter 3, “CPRG Testing of GAL4 Inhibition by GAL80” (Figure 12 & Appendix C):

- Vanessa Di Gioacchino set up the fly crosses
- Laurent Seroude collected the progeny and performed the CPRG assays
- Vanessa Di Gioacchino and Laurent Seroude analyzed the data
ACKNOWLEDGEMENTS

I would first and foremost like to thank Dr. Laurent Seroude for the opportunity to work on such an interesting and challenging project for the past three and a half years and for your guidance throughout. You are a fantastic supervisor who always has time for his students and encourages independent and critical thinking from day one. The skills I have gained in this lab go beyond science and for that I am truly grateful. Thank you to my fellow Seroude Lab members as well – your help in the lab was greatly appreciated and our (lengthy) lab meeting discussions will be missed!

I would also like to thank my Committee members, Dr. Kenton Ko and Dr. Eric Dumont, for your advice, support and thoughtful feedback during this process.

Thank you to Josh Powles, Julie Choi, and Michal Pyc for all the serious and not-so-serious lunchtime conversations and comic relief during the past few years. It’s been fun going through grad school with you!

Last, but far from least, a huge thank you to my parents, my sister, Marcus Seegmiller, and the entire Di Gioacchino and Bortolussi families. Your love, support and encouragement mean the world to me and I couldn’t have done this without it.
# TABLE OF CONTENTS

ABSTRACT ................................................................................................................................. ii
CO-AUTHORSHIP ..................................................................................................................... iv
ACKNOWLEDGEMENTS ............................................................................................................. v
LIST OF TABLES ....................................................................................................................... ix
LIST OF FIGURES .................................................................................................................... x
LIST OF ABBREVIATIONS ......................................................................................................... xi

CHAPTER 1: INTRODUCTION & LITERATURE REVIEW .................................................... 1

*Drosophila, Mental Illness, & Haloperidol* ............................................................................ 1
*Drosophila & Mental Illness* .................................................................................................. 2
*Haloperidol & Dopamine* ..................................................................................................... 3
*DJ858 & Goα47A* .................................................................................................................. 5

*G-Protein Signalling in Drosophila* ..................................................................................... 6
*Goα in Drosophila* ................................................................................................................ 8
*The UAS/GAL4 System* ......................................................................................................... 9
*The GAL80 System* .............................................................................................................. 10

*Objective & Hypothesis* ....................................................................................................... 11

CHAPTER 2: MATERIALS & METHODS ............................................................................ 15

*Fly Stocks* ............................................................................................................................. 15
*Cloning* ............................................................................................................................... 15
*RNA Extraction* .................................................................................................................. 15
*Reverse Transcription* ......................................................................................................... 16
*Polymerase Chain Reaction* ................................................................................................ 16
*Preparation of Insert & Vector* ............................................................................................ 17
*Ligation & Transformation* ................................................................................................ 18
*DNA Isolation & Analysis of Clones* ................................................................................... 18

*Generation of Transgenic Strains* ....................................................................................... 19
*Preparation of DNA for Transformation* ........................................................................... 19
Transformation of Drosophila melanogaster ................................................................. 20
Mapping Insertion Locations ......................................................................................... 20
Establishment of VD Strains .......................................................................................... 21
Establishment of DJ858; VDx3x and DJ858; 3.3+1077 .................................................. 22
Investigating the Crumpled-Wing Phenotype ............................................................... 22
Preparation of Wings for Microscopy .............................................................................. 22
CPRG Assays .................................................................................................................. 23
Fly Crosses ...................................................................................................................... 23

CHAPTER 3: RESULTS ...................................................................................................... 26
Cloning ............................................................................................................................ 26
Cloning of pVD2 ............................................................................................................ 26
Sub-cloning of pVD4 ........................................................................................................ 28
Generation of Transgenic Strains .................................................................................. 28
Injection .......................................................................................................................... 28
Generation & Mapping of UAS-VD Strains ................................................................. 29
Establishment of DJ858; VDx3x Strains ........................................................................ 29
Investigating the Crumpled-Wing Phenotype ............................................................... 31
Interaction with TM3, Sb Balancer ................................................................................ 32
CPRG Testing of GAL4 Inhibition by GAL80 ............................................................... 33
Establishment of DJ858; 3.3+1077 ............................................................................... 35
Preventing the Crumpled Wing Phenotype .................................................................. 35

CHAPTER 4: DISCUSSION ............................................................................................... 48
UAS-Goα Transgenes are Functional ............................................................................ 48
Goα Overexpression Phenotypes .................................................................................. 49
The Crumpled Wing Phenotype ................................................................................... 49
Lethality Caused by Overexpression of UAS-VD Transgenes ...................................... 50
Interaction with TM3, Sb Balancer ................................................................................ 52
GAL80 Prevents the Crumpled Wing Phenotype ......................................................... 54
Significance & Implications for the Future .................................................................... 55
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>LITERATURE CITED</td>
<td>57</td>
</tr>
<tr>
<td>APPENDIX A: DJ858 Phenotype Confirmation</td>
<td>63</td>
</tr>
<tr>
<td>APPENDIX B: Cloning Strategies</td>
<td>64</td>
</tr>
<tr>
<td>APPENDIX C: CPRG Assay Replicate</td>
<td>66</td>
</tr>
</tbody>
</table>
LIST OF TABLES

Table 1 ............................................................................................................. 41
Independent transgenic strains generated from transformations with pVD4 (UAS-VD1) and pVD3 (UAS-VD2)

Table 2 ............................................................................................................. 43
Results showing lethality caused by overexpression of the UAS-VD transgenes
LIST OF FIGURES

Figure 1 ......................................................................................................................... 13
Diagram of the location of the DJ858 insertion within the genome

Figure 2 ......................................................................................................................... 14
The UAS/GAL4 and GAL80 systems for gene manipulation in Drosophila

Figure 3 ......................................................................................................................... 24
Scheme used to generate double homozygous strain DJ858; VDx3x

Figure 4 ......................................................................................................................... 25
Scheme used to generate double homozygous strain DJ858; 3.3+1077

Figure 5 ......................................................................................................................... 37
Cloning strategy for pVD2

Figure 6 ......................................................................................................................... 38
Cloning strategy for pVD4

Figure 7 ......................................................................................................................... 39
Cloning results for pVD2

Figure 8 ......................................................................................................................... 40
Cloning results for pVD4

Figure 9 ......................................................................................................................... 42
Comparison between a wild-type wing and a crumpled wing

Figure 10 ...................................................................................................................... 44
Scoring of DJ858-GAL4/+; UAS-VD/+ progeny for the “crumpled wing” phenotype

Figure 11 ...................................................................................................................... 45
Scoring of DJ858-GAL4/+; UAS-VD/TM3, Sb progeny for the “crumpled wing” phenotype

Figure 12 ...................................................................................................................... 46
CPRG assay comparing LacZ reporter activity for DJ858-GAL4/Bg2 (UAS-LacZ), DJ858-GAL4/Bg2; 1077/+ and DJ858-GAL4/Bg2; 3.3+1077/+.

Figure 13 ...................................................................................................................... 47
Scoring of DJ858-GAL4/+; UAS-VD/3.3+1077 progeny for the crumpled wing phenotype
LIST OF ABBREVIATIONS

2TY  (or 2 x TY or 2 x YT) 2x tryptone and yeast extract
BAR  Bin-Amphipysin-Rvs (protein domain)
BSA  bovine serum albumin
cDNA  complementary deoxyribonucleic acid
CPRG  chlorophenol red-β-D-galactopyranoside
DMIM  *Drosophila* Missing-in-Metastasis
DNA  deoxyribonucleic acid
dNTP  deoxyribonucleotide triphosphate
DSM  Diagnostic and Statistical Manual of Mental Disorders
GDP  guanosine diphosphate
GPCR  G-protein coupled receptor
GTP  guanosine triphosphate
NEB  New England Biolabs
ORF  open reading frame
PCR  polymerase chain reaction
PTX  pertussis toxin
RGS  regulator of G-protein signalling
RNA  ribonucleic acid
TAE  tris-acetate-EDTA
UAS  upstream activation sequence
CHAPTER 1
INTRODUCTION & LITERATURE REVIEW

_Drosophila, Mental Illness, & Haloperidol_

Mental illness encompasses a wide variety of disorders that affect diverse subsets of the population. Despite their prevalence, the underlying causes of many of these illnesses are still poorly understood. For example, schizophrenia affects approximately 1% of the population (Carpenter and Buchanan, 1994) and is characterized by a combination of symptoms including delusions, hallucinations, and disorganized behaviour (Tandon et al., 2013). However, the cause of schizophrenia is unknown and only hypotheses as to the potential causes exist. One hypothesis is that abnormal dopamine activity contributes to schizophrenic symptoms (Madras, 2013). Another hypothesis is a neurodevelopmental one, where a change in the expression of the Wingless (Wnt) signalling pathway results in a synaptic disorganization in the brain, leading to the pathology of schizophrenia (Panaccione et al., 2013). It is possible that abnormal neurotransmitter activity, abnormal brain development, or both, could play a role in the etiology of mental illnesses like schizophrenia. Further research into the genetics behind mental illnesses and the pharmaceuticals used to treat them could help elucidate the causes of these diseases.

The diagnoses of mental illnesses rely on ambiguous psychiatric evaluations through the use of DSM checklists during conversations with patients (Tandon et al., 2013), as opposed to objective, biological testing. Following evaluation, treatment of mental illnesses is often a cycle of trial-and-error of various medications that could cause unpleasant side effects and result in discontinuation of treatment (Vieta et al., 2005). A better understanding of the physiology of these diseases could lead to objective diagnoses and targeted treatments, which in turn could lead to an increased quality of life for patients.
**Drosophila & Mental Illness**

The common fruit fly, *Drosophila melanogaster*, is a proven model organism for the study of human diseases due, in part, to its high genetic tractability. Not only has its genome been fully sequenced and annotated, but there have been a wide array of genetic tools developed specifically for this organism, including balancer chromosomes, and techniques for mutagenesis and gene expression manipulation (Duffy, 2002; Muller, 1927).

Studying the biology and physiology of the fruit fly has led to significant contributions to the fields of human health and medicine (Bellen et al., 2010). Functional orthologs of the majority of human genes can be found in the *D. melanogaster* genome, with greater than 80% similarity in catalytic and other important domains (Bernards and Hariharan, 2001; Rubin, 2000). However, more than having genes and protein functions in common, humans and fruit flies share intricate pathways that can result in more complex phenotypes, such as behaviours. For instance, fruit flies have been shown to be capable of learning and decision-making (Dudai et al., 1976; Yang et al., 2008). There are also several examples of similar responses in fruit flies and in humans to various drugs, including cocaine- and ethanol-induced behaviours and sensitization (McClung and Hirsh, 1998; Moore et al., 1998; Sax and Strakowski, 2001).

The central nervous systems of humans and flies may differ in size and complexity, but both are made up of neurons that develop and function under the same basic principles, using many of the same neurotransmitters (Shulman et al., 2003; Tessier-Lavigne and Goodman, 1996). Furthermore, in the same way that the human brain degenerates, so does that of the fruit fly (Min and Benzer, 1997). Indeed, flies are being used to model and investigate a number of human neurodegenerative diseases, such as Parkinson’s Disease, Huntington’s Disease, Adrenoleukodystrophy, and Spinocerebellar Ataxia (Feany and Bender, 2000; Jackson et al.,
1998; Min and Benzer, 1999; Warrick et al., 1998). These findings lay the groundwork for neuropharmacological research in fruit flies, with future applications for accurate, objective diagnoses and early, targeted treatments for mental illness.

**Haloperidol & Dopamine**

Haloperidol is a first generation, antipsychotic drug used to treat the positive symptoms of schizophrenia, as well as psychosis and behavioural symptoms of other disorders such as bipolar disorder, and autism (Anderson et al., 1989; Leucht et al., 2008; Vieta et al., 2005). However, despite frequently being prescribed and being the subject of clinical and laboratory research, the exact mechanism behind haloperidol’s ability to alleviate the patient’s symptoms remains unresolved (Fasulo and Hemby, 2003; Madras, 2013). The high affinity of haloperidol and other antipsychotics for D2 dopamine receptors has been the main tenet behind the dopamine hypothesis of schizophrenia (Madras, 2013), despite their high affinities for other kinds of receptors as well (Arvanov et al., 1997; Cohen and Lipinski, 1986; Tam and Cook, 1984). Haloperidol has also been shown to affect neural plasticity and changes in gene expression in certain regions of the brain (Fasulo and Hemby, 2003; Panaccione et al., 2013), indicating that there could also be a developmental contribution to schizophrenia.

A recent study altered dopamine signalling in developing flies that, as adults, were used to model schizophrenia using a visual response phenotype (Calcagno et al., 2013), presumably modeling the visual hallucinations experienced by some patients with schizophrenia (Tandon et al., 2013). However, in this study, haloperidol did not induce a change in the phenotype being measured. This is further evidence that haloperidol and dopamine are not necessarily acting on the same pathways. It is possible that a haloperidol-affected pathway exists that acts
independently of, or only has indirect effects on, dopamine signalling.

A single gene mutation could alter a fruit fly’s response to haloperidol, for example, by making them resistant or sensitive to the drug’s effects. Identifying the genes that are mutated may provide insight into how the fly’s nervous system responds to antipsychotics. Some of the genes identified may be involved in drug metabolism, for example, (Llerena et al., 1992) rather than a neurological response to the drug, the latter of which would contribute much more to the understanding of antipsychotic drugs. Mammalian orthologs of the *Drosophila* genes that are involved in a putative haloperidol-affected pathway could eventually be targets for objective diagnoses and treatments.

When wild-type *D. melanogaster* are fed 5mg/ml haloperidol, there is a significant decrease in longevity (Appendix A). A library of *D. melanogaster* mutant strains, which were generated through random mutagenesis using a P-element transposon, was screened for longevity-based mutant phenotypes when being fed haloperidol. This screen found a number of resistant and sensitive strains. Mapping of those mutations identified a variety of candidate genes that might be involved in the response to haloperidol. DJ858 is located in the middle of Goα47A, which encodes an α-subunit of a heterotrimeric G-protein (de Sousa et al., 1989; Schmidt et al., 1989; Thambi et al., 1989; Yoon et al., 1989). This gene is necessary for cell fate and polarity, and is expressed in the nervous system (Katanaev et al., 2005; Wolfgang et al., 1991). DJ1056 is located at the 5’ end of synj (Synaptojanin), which encodes a phosphotidylinositol phosphatase that is involved in clathrin-mediated endocytosis (Verstreken et al., 2003). DJ871 is located in the 5’ end of dmim (Missing-in-Metastasis), which encodes an I-BAR (inverse Bin-Amphiphysin-Rvs) domain protein (Quinones et al., 2010) that is involved in cell migration. Since mental illnesses are likely disorders of the brain, genes that are expressed in the nervous
system (St. Pierre et al., 2014) are reasonable candidates for involvement in mental disorders.

**DJ858 & Goa47A**

DJ858 was identified as resistant to haloperidol; the location of the insertion was mapped using plasmid rescue and was found to be in an intronic region of Goa47A (CG2204), which encodes the “o”-alpha subunit of a G-protein (Figure 1). Cyp49a1 (CG12894), which encodes a member of the Cytochrome P450 protein family (Choi, 2008; St. Pierre et al., 2014), is located approximately 5kb downstream from the insertion.

Goa47A produces multiple transcripts, has at least two different promoters, and is subject to alternative splicing (Fremion et al., 1999; Thambi et al., 1989; Wolfgang et al., 1991; Yoon et al., 1989). Northern blot analysis has shown three different transcript sizes: one larger than 5kb and two that are clearly shorter (Thambi et al., 1989; Yoon et al., 1989). Nine different transcripts can be identified from cDNA libraries (Figure 1) (Adams et al., 2000; St. Pierre et al., 2014; Thambi et al., 1989; Yoon et al., 1989). However, sequence analysis indicates that only 2 proteins are produced, which differ from each other by 7 of the 21 N-terminal amino acids (Adams et al., 2000; Yoon et al., 1989). The expression of some, but not all, of these transcripts is reduced in DJ858 mutants (Wood, 2010), which is evidence that its expression is being affected by the mutation and indicates that Goa47A should be prioritized over Cyp49a1 for further investigation. A genetic rescue will be used to confirm that the decrease in expression of Goa47A is responsible for the haloperidol-resistant phenotype.

With so many different transcripts, it is difficult to determine what kind of construct to use to attempt this rescue. It is possible that there is one specific transcript that needs to be present to reverse this phenotype, or even a specific combination of multiple transcripts. This
introduces a large number of permutations that could potentially be tested. However, a previous study has rescued a deficiency of this gene with a single open reading frame (ORF1 in Figure 1) (Fremion et al., 1999). Thus, it is reasonable and more straightforward to emulate this strategy and use the open reading frames instead of the full cDNA transcripts. Because studies of this gene up to this point have used the same single cDNA construct corresponding to the first of two different ORFs (Devambez et al., 2013; Fremion et al., 1999; Katanaev et al., 2005; Katanayeva et al., 2010; Schwabe et al., 2005), the differential functions of the two Gα isoforms are unknown. Therefore, the rescue will be attempted separately with each ORF in order to determine whether a difference in function can be found.

The promoters for this gene are undefined and the previous rescue was performed with the transgene under the control of the UAS/GAL4 system (described in more detail in a later section), rather than the native promoter (Fremion et al., 1999). Therefore, a UAS promoter will also be used in this experiment. However, it is important to ensure that the artificial promoter will drive expression in as similar a pattern to the native Gα47A promoter as possible. The DJ858 mutation is caused by the insertion of a GAL4 enhancer trap (Brand and Perrimon, 1993; O'Kane and Gehring, 1987). Enhancer traps do not always perfectly replicate the expression of the genes they are near, but previous work has shown that LacZ expression driven by the DJ858 enhancer trap shows a very similar pattern to previously published expression data for Gα47A (Wolfgang et al., 1990; Wood, 2010).

**G-Protein Signalling in Drosophila**

G-proteins are heterotrimeric proteins made up of an α, β, and γ subunit. Gα subunits are the largest of the three subunits and can belong to four classes: Gai/o, Gas, Gq and G12/13
(Malbon, 2005). Ga subunits are fatty-acylated and Gγ subunits are isoprenylated; as a result, these two subunits are bound to the plasma membrane (Casey, 1995). Heterotrimeric G-proteins interact with heptahelical, transmembrane receptors (also known as G-protein-coupled receptors or GPCRs) to activate downstream effectors in a variety of signalling pathways.

GPCRs can respond to a wide variety of ligands including neurotransmitters and macromolecules. The binding of a ligand causes the receptor to undergo a conformational change, initiating its activity as a guanine nucleotide exchange factor (GEF) where the receptor exchanges a GDP molecule bound to the G-protein’s α-subunit for GTP. The Ga-GTP subunit then dissociates from the Gβγ subunit and both moieties are then able to interact with other proteins to effect downstream signals. The Ga subunit has an intrinsic GTPase activity, allowing it to hydrolyse the bound GTP to GDP. The GTPase activity of Ga can occur on its own or be catalyzed by members of the RGS (regulators of G-protein signalling) protein family. Following this hydrolysis, the Ga subunit will usually re-associate with the Gβγ subunit and be ready to transduce the GPCR signal again (Malbon, 2005).

_Drosophila melanogaster_ have 6 genes that encode Ga subunits: Gaαs, Gaαi, Gaαo, Gaαq, Gαf, and concertina; 3 genes that encode Gβ subunits: Gβ5, Gβ13F, and Gβ76C; and 2 genes that encode Gγ subunits: Gγ1 and Gγ30A (St. Pierre et al., 2014). The number of possibilities available for each subunit in a _D. melanogaster_ G-protein means that, theoretically, there are 36 possible heterotrimers. However, with approximately 160 genes encoding GPCRs in _Drosophila_ (Bendena et al., 2012), there must be something other than the identity of the heterotrimer’s subunits that directs the specificity between GPCRs, G-proteins, and their downstream effectors. However, the specificity of G-protein signalling is still not well characterized.

G-proteins are involved in many developmental processes and, as such, null mutations in
these genes are often lethal. When this is the case, it is difficult to elucidate the signalling pathways in which they may act. However, some of the many processes known to be mediated by G-proteins in *D. melanogaster* are cardiogenesis (Fremion et al., 1999), gastrulation (Parks and Wieschaus, 1991), neuronal development and asymmetrical cell division (Schaefer et al., 2001), and the Wingless (Wnt) and Planar Cell Polarity pathways (Katanaev et al., 2005).

**Goα in Drosophila**

*Goα47A* has been shown to be involved in a number of cellular processes in *Drosophila*, including the formation and maintenance of the blood-brain barrier, central nervous system development, heart development, and the Wingless pathway, which is involved in cell fate and cell polarity (Fremion et al., 1999; Katanaev et al., 2005; Schwabe et al., 2005). A different allele of the human ortholog of Goα has been associated with a subset of Japanese schizophrenia patients (Tani et al., 2001). The expression level of this gene is reduced in a sample of American schizophrenia patients (Vawter, 2004).

The subunits of G-proteins are transducers along cell signalling pathways. Identifying the receptor coupled to Goα in DJ858 flies would help narrow down the location and mechanistic effect of this mutation. Goα has been found to transduce the signals of four different G-protein coupled receptors (GPCRs): Moody, DmXr, Octβ1R, and Frizzled. Moody is involved in the development and active maintenance of the blood-brain barrier in fly embryos, and plays a role in cocaine sensitivity (Schwabe et al., 2005). DmXR is found in gustatory receptor neurons and is important for the detection of L-canavanine, a toxic compound produced by plants (Devambez et al., 2013). Octβ1R is an octopamine receptor that plays a role in synaptic bouton formation and larval starvation response (Koon and Budnik, 2012). Finally, Frizzled (Fz) is involved in
both the canonical Wingless (Wnt) pathway, which signals through β-catenin to control cell fate and proliferation, and the non-canonical Planar Cell Polarity pathway, for which the ligand is unknown (Katanaev et al., 2005). All four of these receptors are expressed in the nervous system. One or all of these receptors could be coupled to the specific Goα isoform being affected. Confirming which associations are involved in the response to haloperidol in flies can be worked out relatively quickly. Since mutants for these receptors already exist, double mutants can be generated easily, allowing for the exploration of interactions between various receptors and Goa47A in the response to haloperidol.

The UAS/GAL4 System

Various genetic tools are available for manipulating gene expression in Drosophila, one of which is the UAS/GAL4 system. This bipartite system was found in Saccharomyces cerevisiae and consists of GAL4, a yeast DNA-binding transcription factor, and its binding site, UAS (upstream activating sequence). When introduced into organisms like Drosophila (Fischer et al., 1988), the activity of GAL4 can drive the expression of a responder gene under the control of UAS (Figure 2A).

The GAL4/UAS system in Drosophila consists of two separate transgenic constructs: the driver and the responder. The responder transgene consists of the gene of interest with an otherwise transcriptionally-inactive promoter containing multiple UAS sequences, to which GAL4 can bind and initiate transcription. The driver construct expresses GAL4 under the control of either a tissue-specific promoter (Fischer et al., 1988) or an enhancer trap (Brand and Perrimon, 1993). In an enhancer trap, the expression of GAL4 is under the control of a minimal promoter, which produces a negligible level of GAL4 expression on its own. However, once
inserted into the genome, this promoter is up-regulated by genomic enhancers that usually act on endogenous promoters. Theoretically, an enhancer trap mimics the expression pattern of endogenous genes, but this has to be confirmed for each enhancer trap produced. There are a number of GAL4 strains for which the expression pattern during aging is known (Seroude, 2002; Seroude et al., 2002), so the appropriate driver can be selected as needed for each experiment. This allows the regulation of GAL4 expression in a spatially- and temporally-specific manner.

Strains with each transgene are maintained separately and when the two lines are crossed, progeny carrying both are generated (Figure 2A). This allows for UAS strains with lethal or toxic transgenes to be obtained and maintained without ill effect on the flies until they are crossed with a GAL4 strain that induces expression of the gene of interest.

The GAL80 System

Despite of its wide applicability, the UAS/GAL4 system lacks a level of temporal control over GAL4's activity. The GAL80 system was developed to address this problem by allowing GAL4 to be activated or repressed as needed (Figure 2B).

GAL80 is a protein from S. cerevisiae that represses GAL4 activity by binding to its activation domain and preventing it from interacting with transcriptional machinery. This repression has been shown to occur in Drosophila as well, with no noticeable morphological or behavioural effects caused by the ubiquitous expression of GAL80 (Lee and Luo, 1999). GAL80 has been placed under the control of a minimal promoter containing tetracycline operator sequences (TetO). tTA is a hybrid protein combining the E. coli tetracycline repressor protein and the activation domain from the Herpes Simplex Virus VP16 protein (Gossen and Bujard, 1992) that can bind to TetO and activate transcription of GAL80. When GAL80 is expressed in
UAS/GAL4 flies, expression of the gene of interest is reduced (Figure 2B) (Lee and Luo, 1999). If tetracycline is fed to UAS/GAL4/GAL80 flies, a conformational change is induced in tTA, preventing it from binding to TetO, and in turn preventing transcription of GAL80. This leads to the restoration of the initial expression level of the GAL4-driven, UAS-controlled gene of interest.

If tTA is ubiquitously expressed, this system could be used with any GAL4 driver. tTA has been placed under the control of the α-1 tubulin promoter (Lee and Luo, 1999; Poirier, 2008) and the actin-5c promoter (Stebbins et al., 2001). It has been shown that both transgenes are needed at the same time in order to provide truly ubiquitous expression (Poirier, 2008). Two copies of each transgene are needed to achieve complete repression of GAL4 expression (Poirier, 2008). However, in the context of this experiment, the GAL80 system is used to achieve reduced expression, not full repression, of the genes of interest.

**Objective & Hypothesis**

The location of the P-element in an intron of Goα47A and the reduced expression of certain transcripts of this gene in DJ858 flies indicate that this gene is affected by the insertion mutation. The human ortholog of Goα was found to be significantly reduced in schizophrenia patients compared to unaffected relatives; a mutant allele of the human ortholog of this gene has also been found at a significantly higher frequency in a subset of schizophrenia patients compared to the unaffected population. Therefore it is hypothesized that the insertion has created a mutant allele of Goα47A, which is responsible for the haloperidol-resistance phenotype. The objective of this study is to test that hypothesis using a genetic rescue. However, because the gene encodes two ORFs, it is unclear which is being affected by the mutation. RNA expression
data (Wood, 2010) suggests that expression of transcripts encoding ORF1 is reduced in mutant flies. However, the location of the DJ858 insertion is approximately 60bp upstream of the start of transcription for mRNA corresponding to ORF2 (Figure 1), which indicates that the insertion likely affects ORF2, even if the effect is not detectable by semi-quantitative methods. Therefore, a rescue experiment that uses each ORF separately could help narrow down the effects of the DJ858 mutation, and possibly indicate whether there is a functional difference between the two proteins.
Figure 1: Diagram of the location of the DJ858 insertion (red circle) within the genome – specifically, in an intron of Goα47A (green boxed arrow indicates the gene, green boxes indicate exons of the nine identified Goα47A transcripts). Start of translation for each ORF and end of translation are indicated by blue circles. Primers used for cloning of ORF1 (VD7 & VD2) and ORF2 (VD3 & VD4) are indicated by purple arrows. Nearby gene Cyp49a1 is indicated by the orange boxed arrow.
**Figure 2:** The UAS/GAL4 and GAL80 systems in *Drosophila*. 

**A)** The expression of GAL4 is regulated by endogenous enhancers. In turn, GAL4 binds to UAS and activates the expression of a gene of interest in a tissue- and age-specific pattern. Animals with only a GAL4 or UAS construct will not express the target gene; a fly needs to be carrying both of the transgenes in order to activate expression of the gene of interest. 

**B)** In the absence of tetracycline, GAL80 binds to GAL4, preventing GAL4 from binding to UAS and reducing the level of expression of the gene of interest. In the presence of tetracycline, GAL80 cannot bind to GAL4 and expression occurs as in (A).
CHAPTER 2
MATERIALS & METHODS

Fly Stocks

The strains used in these experiments are:

• $w^{1118}$ (w[1118]; +; +) (Bloomington Fly Stock Center, Indiana University, Stock # 3605)

• DJ858 (w[1118]; P{w[+mW.hs]=GawB}DJ858) (Seroude et al., 2002)

• 2475 (w*; T(2;3)ap$^X_a$, ap$^X_a$/CyO; TM3, Sb$^1$) (Bloomington Fly Stock Center, Indiana University, Stock # 2475)

• $yw, drd^1/Fm7c$ (yw, drd$^1$/Fm7c) (Buchanan and Benzer, 1993)

• Bg2; 3.3+1077 (w; P{w+mC=UAS-lacZ.B}Bg4-1-2; P{w+[mW.hs],[act5c-tTA;TetO-GAL80]Tr3.3=pDJ147}3.3, P{w+[mW.hs],[α1t-tTA;TetO-GAL80]T3.3a=pDJ146}DJ1077) (Poirier, 2008)

• Bg2; 1077 (w; P{w+mC=UAS-lacZ.B}Bg4-1-2; P{w+[mW.hs],[α1t-tTA;TetO-GAL80]T3.3a=pDJ146}DJ1077) (DeVeale, 2004)

• Bg2 (w; P{w+mC=UAS-lacZ.B}Bg4-1-2) (St. Pierre et al., 2014)

All stocks were maintained at 18 or 25°C on fresh *Drosophila* food (0.01% molasses, 8.2% cornmeal, 3.4% yeast extract, 0.94% agar, 0.18% benzoic acid and 0.66% propionic acid).

Cloning

RNA Extraction

Total RNA was extracted from $w^{1118}$ flies using TRIzol Reagent (Invitrogen), following the protocol as previously described (Zheng et al., 2005). Once extracted, a 200x dilution of the
RNA was quantified using disposable microcuvettes (UltiDent) and a SpectraMax Plus 384 spectrophotometer (Molecular Devices) at 260nm, with 20ng/µl yeast tRNA as a standard. 7µg of the RNA were run on a 0.7% agarose TAE gel to visually determine the quality of the RNA. The ladder used on all agarose TAE gels was 1kb GeneRuler plus (Thermo Scientific).

Reverse Transcription

25µg of total RNA were reverse transcribed in a final volume of 15µl, using the protocol for SuperScript II Reverse Transcriptase (Invitrogen) and poly-dT primers. Successful reverse transcription (RT) was confirmed by PCR using DCP-1 primers (Zheng et al., 2005) and 100x-diluted, 10x-diluted and undiluted cDNA. For these reactions, each 25µl reaction volume included 1µl of the RT template, 200nM of each primer, 200µM dNTPs (Roche), 1 unit of Taq DNA Polymerase (NEB), 1x ThermoPol Buffer (NEB). The reaction was run under the same conditions used in previous studies (Zheng et al., 2005).

Polymerase Chain Reaction

The sequence of the primers used were VD7 (forward) 5’-TATCTCGAGCTGCAGAAAAGCCCCGTGTAATTC-3’ and VD2 (reverse) 5’-TATCCGCGGAATCTTAGGGTTGGGCATCG-3’. PCR using the VD7-VD2 primer pair was done in 25µl reactions containing 1µl of 10x diluted cDNA, 500nM each primer, 200µM dNTPs, 1u Phusion High-Fidelity DNA Polymerase (NEB), 1x Finn HF Buffer (NEB). The reaction was run in a T-Gradient Thermoblock (Biometra) under the following conditions: a single pre-PCR cycle at 98˚C for 3min, 32 cycles of denaturation at 98˚C for 15s, hybridization at 55˚C for 30s, and elongation at 72˚C for 15s, followed by a single post-PCR cycle at 72˚C for 10min. A
temperature gradient was used to determine the optimal hybridization temperature of 55°C. The VD7-VD2 PCR product is 358bp long.

**Preparation of Insert & Vector**

The insert for pVD2 was prepared by digesting 400ng of the VD7-VD2 PCR product with 40u each of XhoI (NEB) and AccI (NEB) in 1x NEBuffer 4 supplemented with BSA (NEB). The vector was prepared by digesting 2μg of pVD1 (Di Gioacchino, 2012) with 40u each of XhoI (NEB) and AccI (NEB) in 1x NEBuffer 4 supplemented with BSA (NEB), then dephosphorylating it with 10u of Antarctic Phosphotase (NEB).

The insert for pVD4 was prepared by digesting 4μg of pVD2 with 100u each of PstI and BamHI in 1x BamHI NEBuffer supplemented with BSA (NEB). The pINDY5 vector was prepared by digesting 3μg of pINDY5 with 60u of PstI and 40u of BglII in 1x NEBuffer 3 supplemented with BSA (NEB).

All reactions were run on a 0.5% agarose TAE preparative gel. The bands corresponding to the insert and vector were each excised from the gel and purified using the QIAquick (Qiagen) kit. The VD7-VD2 insert was eluted with 25μl of elution buffer and the pVD1 vector was eluted with 50μl of elution buffer. The pVD2 insert was eluted with 35μl of elution buffer and the pINDY5 vector was eluted with 30μl of elution buffer. 2μl each of the insert and vector were run on a 0.7% agarose TAE gel to confirm the purification was successful and to compare relative concentrations.
**Ligation & Transformation**

For each ligation, insert and vector were combined in a 10µl reaction containing 400u of T4 DNA Ligase (NEB) and 1x supplied T4 DNA Ligase Buffer (NEB). For the ligation to generate pVD2, 6.5µl of the VD7-VD2 insert and 1.5µl of the pVD1 vector were used. For the ligation to generate pVD4, 5µl of the pVD2 insert and 2µl of the pINDY5 vector were used. The reactions were left at 16°C overnight.

5µl of the ligation reaction was added to 40µl of electro-competent XL1 Blue *Escherichia coli*. Using a GenePulse (BioRad) electroporator, samples were pulsed at 1.5V. 500µl of 2TY media were added to the bacteria immediately following the pulse. The bacteria were allowed to recover at 37°C with agitation for one hour. All 545µl of the culture were plated onto 2TY plates supplemented with 100µg/ml ampicillin and left to grow at 37°C overnight. Positive (1ng pGEM7 DNA), negative (dH₂O), insert-only, and vector-only controls were each transformed separately and plated similarly – except for the positive control, for which 10⁻², 10⁻³, and 10⁻⁴ dilutions were each plated to get a more accurate count of transformed cells. The ampicillin-resistant colonies were counted and randomly chosen colonies from the ligation plate were streaked onto a fresh 2TY + 100µg/ml ampicillin plate, inoculated in 7ml 2TY + 100µg/ml ampicillin, and left to grow overnight at 37°C with agitation.

**DNA Isolation & Analysis of Clones**

Plasmid DNA was purified from 1.5ml of each overnight culture using the QIAprep Spin Mini-prep Kit (Qiagen), according to the supplied protocol. 2µl of each colony’s plasmid DNA were run on a 0.7% agarose TAE gel with a control for size. Clones that migrated at the same rate were digested with restriction endonucleases for further analysis. All digests were done in a
10µl reaction volumes with 5-10u of each enzyme and 1x of the appropriate, manufacturer-supplied buffer. All digests, along with undigested controls, were run on a 0.7% agarose TAE gel.

The pVD2 candidate plasmids and the pVD1 control were each digested with AccI (NEB) and XhoI (NEB) in 1x NEBuffer 4 supplemented with BSA (NEB); this digest was used to look for the presence of the correct vector. The candidate plasmids and pVD1 were also digested with EcoRV (NEB) and BamHI (NEB) in 1x NEBuffer 2 supplemented with BSA (NEB); this digest was used to look for the different-sized inserts used in each plasmid’s respective cloning. pVD2 candidate plasmids with the expected fragment sizes were sent for sequencing (Operon) using the T3 primer.

The pVD4 candidate plasmids and the pINDY5 control were each digested with NotI (NEB) in 1x NEBuffer 3 supplemented with BSA (NEB); this digest was used to look for the presence of the correct vector. The pVD4 candidate plasmids and the pVD2 control were also digested with SphI (NEB) in 1x NEBuffer 2; this digest was used to look for the presence of the correct insert. The final pVD2 and pVD4 were stored as DNA and bacterial stocks.

**Generation of Transgenic Strains**

**Preparation of DNA for Transformation**

pVD3, pVD4 (Di Gioacchino, 2012), and a πΔ2.3 helper plasmid (Robertson et al., 1988) were each used to inoculate a culture in 25ml 2TY + 100µg/ml ampicillin, and left to grow overnight at 37°C with agitation. Each plasmid was isolated from culture using the HiSpeed Plasmid Maxi Kit (Qiagen), following the supplied protocol. A 100x dilution of the DNA was quantified using disposable microcuvettes (UltiDent) and a SpectraMax Plus 384
spectrophotometer (Molecular Devices) at 280nm, with 12.5ng/µl DNA as a standard. 75ng of the DNA were run on a 0.7% agarose gel to visually determine the quality of the DNA. Two DNA mixtures were made, each with the pπΔ2.3 helper plasmid at a concentration of 0.08µg/µl and either pVD3 or pVD4 at a concentration of 0.4µg/µl.

Transformation of Drosophila melanogaster

Transgenic flies were generated by standard methods (Rubin and Spradling, 1982), using the w\textsuperscript{1118} recipient strain. Females were allowed to mate and lay eggs for twenty minutes. 50-60 newly fertilized eggs were aligned using double-sided tape onto a microscope slide, stained with Voltalef oil, and injected into the posterior end with a DNA mixture using a Femtojet microinjector (Eppendorf). All injections were done before the embryos entered the cellular stage. The double-sided tape with the injected embryos on it was removed from the glass slide and placed into a vial containing standard fly food. The injected embryos were allowed to develop into adulthood.

Mapping Insertion Locations

Each adult fly that eclosed after injection (transformant, P) was crossed with a w\textsuperscript{1118} fly of the opposite sex. The coloured-eye progeny (F\textsubscript{1}) of each cross were collected and crossed with a w\textsuperscript{1118} fly of the opposite sex. One male progeny (F\textsubscript{2}) of each eye colour was crossed by a virgin 2475 female. The strain 2475 carries the CyO (curly wings) second chromosome balancer and the TM3, Sb (stubble bristles) third chromosome balancer, both of which are homozygous lethal. Male progeny (F\textsubscript{3}) that had red eyes, curly wings, and stubble bristles were crossed with virgin w\textsuperscript{1118} females. The F\textsubscript{4} progeny were scored for phenotype to determine on which chromosome
each insertion was located. If the insertion in a given transformant was on the second chromosome, red eyes and curly wings would be segregated in the progeny. If the insertion was on the third chromosome, red eyes and stubble bristles would be segregated. If the insertion was on the X chromosome, only female progeny would have red eyes. Finally, if the insertion was on the fourth chromosome, there would be progeny of both sexes with all possible combinations of the three traits.

Establishment of VD Strains

Heterozygous stocks were generated by self-crossing F₃ males and virgin females that had red eyes, curly wings, and stubble bristles and then selecting for F₄ progeny that had red eyes and the balancer that corresponded to the chromosome on which the insertion was located, but not the other balancer. For strains with the insertion on the X chromosome, a male from the F₂ generation was crossed with a virgin Fm7c female. Fm7c carries a Bar (eye shape) X balancer chromosome that is homozygous lethal. A heterozygous stock was generated by crossing virgin female progeny (F₃) that were both red-eyed and bar-eyed with bar-eyed males and selecting for red-eyed males and red-eyed, bar-eyed virgin females. For strains that were homozygous viable, homozygous stocks were generated using the heterozygous stocks and selecting for flies that had no balancers. A homozygous stock of a strain with the insertion on the fourth chromosome was generated by selecting for males and virgin females with darker red eyes; heterozygous flies of that particular strain had eyes that were a lighter shade of red.
Establishment of Double Homozygous Strains (DJ858; VDx3x or DJ858; 3.3+1077)

The process used to generate strains that were homozygous for both DJ858 and a given VD transgene located on the third chromosome (“VDx3x”) is outlined in Figure 3. The process of generating a DJ858; 3.3+1077 stock is outlined in Figure 4.

First, males from a strain carrying an insertion on the third chromosome (either a VDx3x strain or Bg2; 3.3+1077) were crossed to virgin 2475 females. F₁ progeny with red eyes, curly wings, and stubble bristles were self-crossed in order to obtain F₂ flies of the genotype CyO/++; VDx3x or CyO/Bg2; 3.3+1077, which were homozygous for the insertion but also had the CyO balancer. Meanwhile, DJ858 males were crossed to virgin 2475 females. F₁ progeny with red eyes, curly wings, and stubble bristles were self-crossed in order to obtain F₂ flies of the genotype DJ858; TM3, Sb/+, which were homozygous for DJ858 but also had the TM3, Sb balancer.

DJ858; TM3, Sb/+ virgin females were crossed with either CyO/++; VDx3x or CyO/Bg2; 3.3+1077 males. F₃ progeny that had curly wings and stubble bristles (either DJ858/CyO; VDx3x/TM3, Sb or DJ858/CyO; 3.3+1077/TM3, Sb) were self-crossed in order to obtain the double homozygous stock of either DJ858; VDx3x or DJ858; 3.3+1077 flies. For the DJ858; VDx3x scheme, this final cross was used to score progeny in order to measure lethality.

Investigating the Crumpled-Wing Phenotype

Preparation of Wings for Microscopy

Wild-type wings and crumpled wings were dissected from the thorax of the respective fly by the hinge using forceps. The wings were dipped in toluene to remove the waxy coating and placed in a drop of 10% glycerol on a glass microscope slide. Another drop of 10% glycerol was
placed on a cover slip and the cover slip was applied to the sample. Pictures were taken with a Stemi Sv11 microscope (Zeiss), AxioCam HRC camera (Zeiss), and OpenLab software (version 4.0.1).

**CPRG Assays**

Males from Bg2, Bg2; 1077 and Bg2; 3.3+1077 were each crossed with virgin DJ858 females. 5 male and 5 female F1 progeny were collected within 48 hours of eclosion and tested 7 days later, 7-9 days after eclosion. CPRG enzymatic assays were performed as previously described (Poirier, 2008; Seroude et al., 2002).

**Fly Crosses**

To examine the difference in the ability of VD1 and VD2 to cause crumpled wings, males from each independent transgenic VD1 and VD2 strain were crossed with virgin DJ858 females. The progeny were scored for the presence of the crumpled wing phenotype.

To examine the effect of TM3, Sb on the frequency of flies with the crumpled wing phenotype, virgin DJ858; TM3, Sb/+ females were crossed with males from VD131, VD133, VD134, VD231 and VD233. Progeny were scored simultaneously for the presence of the crumpled wing phenotype and stubble bristles.

To examine whether GAL80 could restore crumpled wings to wild-type, virgin DJ858; 3.3+1077 females were crossed with males from VD131, VD133, VD134, VD231 and VD233. The progeny were scored for the presence of the crumpled wing phenotype.

Two-tailed, type-two t-tests and \( \chi^2 \) tests were performed in Microsoft Excel 2011 (version 14.4.4).
Figure 3: Scheme used to generate double homozygous strain DJ858; VDx3x. “CyO” indicates the second chromosome balancer with a curly-wing marker and “Sb” indicates the third chromosome balancer with a stubble bristle marker.
**Figure 4:** Scheme used to generate double homozygous strain DJ858; 3.3+1077. “Bg2” indicates \textit{UAS-LacZ}, “3.3” indicates \(\alpha_{1tubulin-GAL80}\), “1077” indicates \(actin5c-GAL80\), “CyO” indicates the second chromosome balancer with a curly-wing marker, and “Sb” indicates the third chromosome balancer with a stubble bristle marker.
CHAPTER 3
RESULTS

Cloning

The gene Goa47A encodes 9 different transcripts, likely due to a combination of alternative splicing and two different promoters (de Sousa et al., 1989; Schmidt et al., 1989; Thambi et al., 1989; Yoon et al., 1989). Each transcript contains only one of the two open reading frames (ORFs) (Figure 1), which differ from each other only at the 5’ end. ORF2 was previously cloned into a vector that would facilitate sequencing, called pVD1 (Di Gioacchino, 2012). ORF2 was subsequently sub-cloned into a fly injection vector; that plasmid is called pVD3 (Appendix B) (Di Gioacchino, 2012).

The cloning of ORF1 was accomplished by amplifying only the 5’ end of that ORF. The PCR product was used to replace the 5’ end of ORF2 in pVD1 and generate a plasmid called pVD2 (Figure 5). Sequencing confirmed the accuracy of the polymerase, after which the entire ORF1 was sub-cloned into the same injection vector that was used for pVD3, generating a final plasmid called pVD4 (Figure 6).

Cloning of pVD2

A pair of primers was designed to bind to cDNA upstream of the start codon for ORF1 (VD7) and downstream of the point at which the two ORFs become identical (VD2). This PCR fragment (Figure 7A) and pVD1 were digested with restriction endonucleases to create the insert and vector, and the two were ligated together to generate pVD2.

The ligation of the PCR insert to the pVD1 vector was transformed into E. coli with a transformation efficiency of 1.3x10\(^{10}\) transformants/µg of DNA, resulting in 78 ampicillin-
resistant colonies; the vector-only control yielded 6 colonies, the insert control yielded 2 colonies and the negative control yielded 2 colonies. Five colonies were randomly chosen for analysis.

In determining which clone contained the correct sequence, the first factor considered was whether the cloned plasmids were the same size as the expected plasmid, so an agarose gel was used to screen for size. Because the size difference between pVD2 and pVD1 is less than 50bp, pVD1 was used as a positive control. Three of these plasmids, pVD2.3, pVD2.4 and pVD2.5, migrated at the same rate as pVD1 (Figure 7B) and were chosen for further analysis. pVD2.9 migrated a little slower than expected and pVD2.10 migrated much faster, so those two plasmids were likely not the correct pVD2 and were not analyzed further.

In order to determine whether the cloned plasmids contained the desired sequences, the clones and pVD1 were digested using restriction endonucleases. EcoRV and BamHI were used to generate a 563bp fragment that should be shared by the clones and the pVD1 vector. The digests of all three clones, as well as the pVD1 positive control, generated the 563bp fragment in said digest (Figure 7C), indicating that the sequences are the same and the intended vector was likely present. The three clones were also digested by AccI and XhoI to generate a 283bp fragment from the newly inserted 5’ end of ORF1 (Figure 7C). In this case, pVD1 was used as a negative control, where a 349bp fragment was generated corresponding to the 5’ end of ORF2.

All three candidate clones had the expected fragment pattern, so pVD2.4 was selected for the final step of analysis, which was to confirm the exact sequence of the cloned plasmid through sequencing of the newly amplified section of ORF1. The sequence of pVD2.4, when compared to the expected sequence, showed a single point mutation at position -18, with +1 being the first nucleotide of the start codon (Figure 7D). This mutation is outside of the region that flanks the start codon with the most highly conserved nucleotides for Drosophila (Cavener and Ray, 1991).
pVD2.4 was therefore used in the subsequent sub-cloning.

**Sub-cloning of pVD4**

pVD2 and pINDY5 were digested with endonucleases to create the insert and vector, respectively, for pVD4. These two fragments were then ligated together. The ligation of the pVD2 insert to the pINDY5 vector was transformed into *E. coli* with a transformation efficiency of $8.9 \times 10^9$ transformants/µg of DNA, resulting in 1736 ampicillin-resistant colonies; the vector control yielded 3 colonies, the insert control yielded 1 colony, and the negative control yielded 10 colonies. Four colonies – pVD4.1, pVD4.2, pVD4.3 and pVD4.4 – were randomly chosen to be screened for size. pVD3 was used as a positive control, since the size difference between pVD4 and pVD3 is less than 50bp.

All four of these cloned plasmids migrated at the same rate as pVD3 (Figure 8A), so two were randomly chosen for analysis by restriction endonucleases. This analysis showed that both clones, pVD4.1 and pVD4.4, shared a 4.9kb fragment with pINDY5 in a digest by NotI, indicating the presence of the correct vector. The clones also shared a 390bp fragment with pVD2 in a digest by SphI, indicating the presence of the correct insert (Figure 8B). pVD4.4 was therefore used in the *Drosophila* transformation.

**Generation of Transgenic Strains**

**Injection**

A total of 200 embryos were injected with the pVD3+pπΔ2.3 mixture. Of those, 28 survived to adulthood. Each of those adult flies was individually crossed with a $w^{1118}$ fly of the opposite sex; 22 of those adults were fertile and 7 gave at least one red-eyed progeny, indicating
that the parent had \( w^+ \) gametes. This indicates that 3.5% of all embryos that were injected incorporated the construct into their germ cells.

250 embryos were injected with the pVD4+\( p\pi \Delta 2.3 \) mixture and 62 of those survived to adulthood. 43 of those adults were fertile and gave progeny in a cross by \( w^{1118} \); 9 of them gave at least one red-eyed progeny. In this case, 3.6% of embryos that were injected incorporated the construct into their germ cells.

Generation & Mapping of UAS-VD Strains

Eight independent strains were identified within the progeny of the transformants by pVD3 and are referred to as VD2 strains (Table 1). Twelve independent strains were identified from the transformation with pVD4 and are referred to as VD1 strains (Table 1).

Each red-eyed progeny was individually crossed by 2475. Male progeny from that cross with red eyes, curly wings, and stubble bristles were then crossed by virgin \( w^{1118} \) females and the progeny were scored for eye colour, curly wings, and stubble bristles. For each strain, the chromosome on which the insertion was located was identified by looking for segregation of the red eye colour from curly wings, stubble bristles, or male flies.

The first digit in the names of the resulting strains indicates the ORF in the construct with which they were transformed; the second digit indicates the chromosome on which the insertion is located.

Establishment of DJ858; VDx3x Strains

In order to assess whether either of these transgenes could return the haloperidol-resistant phenotype of DJ858 flies to wild-type, flies that are homozygous for DJ858 and heterozygous for
a UAS-VD transgene will be tested since the mutant phenotype is recessive (unpublished data).

Since it has been shown that the expression localization of a UAS reporter gene under the control of DJ858 (Wood, 2010) is similar to that of Goa47A (Wolfgang et al., 1990), DJ858 will be used to drive expression of the UAS-VD transgenes. In order to generate those experimental flies, a strain that is homozygous for both DJ858 and a VD insertion is needed. However, because the DJ858 insertion is located on the second chromosome, VD strains with insertions on the second chromosome were not used, since combining those two genes would require recombination.

The most straightforward way to generate the double homozygous strain was to use VD strains with the insertion located on the third chromosome (“VDx3x”). The strain 2475 would be used in order to take advantage of the fact that it would reduce the number of generations needed in the scheme. Such a stipulation narrowed the choices of UAS-VD strains from 20 down to 8, with 4 strains corresponding to each ORF. The scheme also required that the flies be homozygous viable, so the strains that were ultimately used were VD131, VD133, VD134, VD231, and VD233.

During the process of generating the DJ858; VDx3x strain (Figure 3), a phenotype with variable penetrance was identified. Flies that were heterozygous for both DJ858 and certain VD insertions exhibited a “crumpled wing” phenotype. The wings of these flies appeared normal upon eclosion but never expanded or unfolded into wild-type morphology (Figure 9). This is an indication that the transgene is being expressed, and has been seen with the overexpression of Goa in previous studies (Katanayeva et al., 2010). In addition, the crumpled wings prevent identification of the CyO balancer and therefore the genotype is left unclear. However, enough flies with wild-type, expanded wings were available in order to move forward with the scheme.

After the final cross, flies with no markers – thereby indicating the DJ858; VDx3x flies –
were generally not present amongst the progeny. Therefore, these two insertions together, either DJ858 with VD1 or DJ858 with VD2, are homozygous lethal (Table 2). The exception was males of DJ858; VD231, which were present, but at a lower than expected frequency. However, with no females of that genotype, the desired stock still could not be generated. In the self-crosses with VD131 and VD133, there were also no progeny that were homozygous for just one of the insertions and heterozygous for the other without having crumpled wings. The crosses using VD yielded both DJ858; VD2/TM3, Sb and DJ858/CyO; VD2 progeny. There were also progeny with crumpled wings, but only those that were homozygous for VD2. These crosses were fairly close to showing the expected frequencies in the progeny for each genotype, but there were still a number of progeny with crumpled wings and, again, lethality in the double homozygous progeny.

**Investigating the Crumpled-Wing Phenotype**

Within the subset of VD strains that were used in the DJ858; VDx3x scheme, all three VD1 strains showed the crumpled wing phenotype – to varying degrees of penetrance – when crossed with DJ858, but the two VD2 strains did not. Therefore, all 20 available VD strains were crossed with DJ858 to further investigate this difference between the two ORFs. Some VD strains were not homozygous viable (VD122, VD126, VD135, VD232, and VD234), so a balanced stock had to be used to set up the cross. In these cases, only half of the progeny carried the UAS-VD insertion and only those progeny were scored in this experiment. It should be noted that because males of the VD strains were used in these crosses, male progeny of crosses using the strains with the insertion on the X chromosome (VD111, VD112, VD113, VD211 and VD212) do not carry the UAS-VD insertion.
Scoring the progeny of these crosses showed that seven of the twelve VD1 strains display the crumpled wing phenotype, while only one of the eight VD2 strains does so (Figure 10). Within the seven VD1 strains, there is variable penetrance of the crumpled wing phenotype, but the strains can be grouped according to the level of penetrance. Only a small percentage (approximately 2-3%) of female progeny were affected in the crosses with VD113, VD125, and VD135, while males were not affected. Both male and female progeny from crosses with VD124, VD131, and VD133 were affected, and at slightly higher rates (approximately 2-5% of males and 3-6% of females). Finally VD134 is the only strain for which 100% of progeny that eclosed showed the crumpled wing phenotype. Additionally, VD134 progeny showed a striking level of pupal lethality; only 3-5% of larvae that pupated eclosed as adults, while the rest died as pupae and turned black. Meanwhile, the single VD2 strain that exhibited the crumpled wing phenotype fell into the middle category, where both sexes of progeny were affected, with females (almost 7%) being affected more than males (4.5%).

Interaction with TM3, Sb Balancer

Within these experiments, the crumpled wing phenotype was first noticed during the scheme to generate DJ858; VDx3x flies, in flies that were carrying the TM3, Sb balancer. Non-quantitative observations made of those progeny indicated that the frequency of the crumpled wing phenotype was higher than was measured in the previously described experiment. However, the TM3, Sb chromosome was not present in the previous experiment. Therefore, VDx3x strains were crossed with DJ858; TM3,Sb/+ flies in order to determine whether TM3, Sb has an effect on the frequency of the crumpled wing phenotype.

The frequency of flies with crumpled wings did increase when TM3, Sb was present with
VD131 and VD133 (Figure 11). None of the flies with VD134 that eclosed had stubble bristles, which might indicate that DJ858/+; VD134/Tm3, Sb is even more lethal than DJ858/+; VD134/+. However, the presence of TM3, Sb did not change the fact that crumpled wings are not seen in flies heterozygous for DJ858 and either VD231 or VD233.

In addition to the increased frequency of crumpled wings with TM3, Sb, an unexpected observation was made from the progeny of these crosses. Based on the genotypes of the parents, 50% of the progeny were expected to have wild-type bristles and 50% were expected to have stubble bristles. Interestingly, there were significantly fewer progeny with stubble bristles than expected in all of the crosses with VD1x, except for males with VD131 (VD131 p♂>0.1, p♀<0.001; VD133 p♂<0.001, p♀<0.001; VD134 p♂<0.001, p♀<0.001) (Figure 11). The ratios between the TM3, Sb and wild-type chromosomes were as expected with VD2x, except for females with VD233 (VD231 p♂>0.1, p♀<0.05). These results reflect what has been seen thus far, in that VD1 strains have different phenotypic ratios in their progeny than VD2 strains, and the effects are stronger in females. The results also suggest that there is some kind of interaction between the TM3, Sb balancer and UAS-VD1.

**CPRG Testing of GAL4 Inhibition by GAL80**

In general, two copies of a gene in *D. melanogaster* will result in higher expression than only one copy. This phenomenon can be seen in this study when one copy each of DJ858-GAL4 and UAS-VD1 results in crumpled wings, but two of copies of either or both transgenes can result in lethality. While the presence of crumpled wings indicates that the transgenes are being expressed, the concern remains that crumpled wings may also be an indication that overexpression of Goα above a given threshold is causing poor health in those flies. Since the
intention is to eventually use these flies in a longevity assay to assess whether either transgene can rescue the haloperidol-resistant phenotype, it is important that overexpressing these transgenes does not shorten the flies’ lifespan. Therefore, the ideal situation would be such that the transgenes are being expressed, but at a level where crumpled wings are not seen; this would hypothetically be an indication that the flies are not being negatively affected by the expression of the UAS-VD transgenes.

The UAS-VD transgenes were being overexpressed using the UAS/GAL4 system. When this overexpression occurred, the crumpled wing phenotype was seen. Therefore, if GAL4 was partially inhibited, the expression of the transgene should be reduced, thereby reducing or preventing the crumpled wing phenotype altogether. In order to reduce the amount of GAL4 binding to the UAS promoter, the GAL4 inhibitor GAL80 was used. It has been shown that the level of GAL4 inhibition is related to the number of copies of the GAL80 gene that are present (Poirier, 2008). However, GAL80’s inhibition of GAL4 had not been measured under the control of the DJ858 driver. Therefore, the level of inhibition that one copy or two copies would have, if any, on DJ858-GAL4 had to be measured before introducing GAL80 into the experimental flies.

Virgin DJ858 females were crossed with males from Bg2 (UAS-lacZ), Bg2; 1077 (UAS-lacZ with 1 copy of GAL80), and Bg2; 3.3+1077 (UAS-lacZ with 2 copies of GAL80). Using a CPRG assay, the level of β-galactosidase activity was measured in males and females 7-9 days after eclosion as an indicator of the level of expression of UAS-lacZ (Figure 12 & Appendix C). Male and female DJ858/Bg2; 1077/+ flies did not have a significantly different level of β-galactosidase activity than DJ858/Bg2; + flies of the same sex, indicating that one copy of GAL80 did not significantly reduce the expression of lacZ (p♀=0.35, p♂=0.55). However, β-galactosidase activity in DJ858/Bg2; 3.3+1077/+ flies was reduced by approximately 75% in
males and 50% in females, a significant reduction in \textit{UAS-lacZ} expression (p$^{\varnothing} = 5.0 \times 10^{-12}$, p$^{\varnothing} = 2.6 \times 10^{-8}$).

Since 2 copies of \textit{GAL80} can significantly reduce the expression of a gene under the control of a UAS promoter driven by DJ858, 3.3+1077 should be added to the scheme in order to generate flies that carry both the DJ858 mutation and a \textit{UAS-VD} transgene, but that do not show the crumpled wing phenotype. Such flies could then be used in a rescue experiment.

\textit{Establishment of DJ858; 3.3+1077}

In order to generate flies that had the DJ858 mutation, a \textit{UAS-VD} transgene and 3.3+1077, the 2-copy version of \textit{GAL80}, a double homozygous strain DJ858; 3.3+1077 was needed. This was generated successfully, following the scheme outlined in Figure 4 and was maintained as a stock.

\textit{Preventing the Crumpled Wing Phenotype}

To test whether \textit{GAL80} can sufficiently inhibit \textit{GAL4} in DJ858 flies in order to prevent the crumpled wing phenotype, virgin DJ858; \textit{GAL80} females were crossed with males from the initial subset of \textit{VD} strains that were used (VD131, VD133, VD134, VD231, and VD233). None of the progeny from crosses by VD131, VD133, VD231, and VD233 had crumpled wings (Figure 13), indicating that \textit{GAL80} prevented the crumpled wing phenotype that was seen in DJ858/+; VD131/+ and DJ858/+; VD133/+ flies. For VD134, approximately 14.5% of males and 31% of females with \textit{GAL80} had wild-type wings, whereas 0% of flies without \textit{GAL80} had wild-type wings. In addition, between 6.5-21% of larvae that pupated eclosed as adults with \textit{GAL80} present compared to only 3-5% without \textit{GAL80}. In this case, not only was \textit{GAL80} able
to prevent the crumpled wing phenotype in a good proportion of the flies, but it also reduced the rate of pupal lethality.
Figure 5: Cloning strategy for pVD2.
Figure 6: Cloning strategy for pVD4.
Figure 7: Results from the cloning of pVD2. A) VD7-VD2 PCR product. B) Screen of clones by size, as compared to pVD1. C) Results from the restriction endonuclease analysis of pVD2 clones. Arrows highlight the 349bp band in lane 6, the 283 bp bands in lanes 7-9, and the 563bp band in lanes 10-13. D) Results from the sequencing of pVD2.4. The white background highlights the single nucleotide mismatch between the cloned plasmid (pVD2.4 ORF) and the expected sequence (pVD2 ORF). Light grey boxes show areas where the two sequences are identical; dark grey boxes show the most highly conserved nucleotides in the D. melanogaster consensus translation “initiation sequence” (Cavener and Ray, 1991).
Figure 8: Results from the cloning of pVD4. A) Screen of clones by size, as compared to pVD3. B) Results from the restriction endonuclease analysis of pVD4 clones. Arrows highlight the 4.9kb bands in lanes 3, 5 and 11, as well as the 390bp bands in lanes 6, 9 and 12.
Table 1: Independent transgenic strains generated from transformations with pVD4 (UAS-VD1) and pVD3 (UAS-VD2) and their chromosomal location, viability and status as a stock.

<table>
<thead>
<tr>
<th>Strain Name</th>
<th>Chromosome</th>
<th>Homozygous Lethal</th>
<th>Homozygous Sterile</th>
<th>Homozygous Stock</th>
<th>Balanced Stock</th>
</tr>
</thead>
<tbody>
<tr>
<td>VD211</td>
<td>X</td>
<td>No</td>
<td>No</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>VD212</td>
<td>X</td>
<td>No</td>
<td>No</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>VD221</td>
<td>II</td>
<td>No</td>
<td>No</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>VD231</td>
<td>III</td>
<td>No</td>
<td>No</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>VD232</td>
<td>III</td>
<td>Yes</td>
<td>N/A</td>
<td>N/A</td>
<td>✓</td>
</tr>
<tr>
<td>VD233</td>
<td>III</td>
<td>No</td>
<td>No</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>VD234</td>
<td>III</td>
<td>Yes</td>
<td>N/A</td>
<td>N/A</td>
<td>✓</td>
</tr>
<tr>
<td>VD241</td>
<td>IV</td>
<td>No</td>
<td>No</td>
<td>✓</td>
<td>N/A</td>
</tr>
<tr>
<td>VD111</td>
<td>X</td>
<td>No</td>
<td>No</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>VD112</td>
<td>X</td>
<td>No</td>
<td>No</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>VD113</td>
<td>X</td>
<td>No</td>
<td>No</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>VD121</td>
<td>II</td>
<td>No</td>
<td>No</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>VD122</td>
<td>II</td>
<td>No</td>
<td>Yes</td>
<td>N/A</td>
<td>✓</td>
</tr>
<tr>
<td>VD124</td>
<td>II</td>
<td>No</td>
<td>No</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>VD125</td>
<td>II</td>
<td>No</td>
<td>No</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>VD126</td>
<td>II</td>
<td>Yes</td>
<td>N/A</td>
<td>N/A</td>
<td>✓</td>
</tr>
<tr>
<td>VD131</td>
<td>III</td>
<td>No</td>
<td>No</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>VD133</td>
<td>III</td>
<td>No</td>
<td>No</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>VD134</td>
<td>III</td>
<td>No</td>
<td>No</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>VD135</td>
<td>III</td>
<td>No</td>
<td>Yes</td>
<td>N/A</td>
<td>✓</td>
</tr>
</tbody>
</table>
Figure 9: Comparison between (A) a wild-type wing (32x magnification, scale bar = 0.4mm) and (B) a crumpled wing (64x magnification, scale bar = 0.2mm).
Table 2: Percentage of progeny from each self-cross of DJ858/CyO; VDx/TM3, Sb that were a given genotype. “Crumpled wing” columns indicate percentage of progeny that had crumpled wings and could either be heterozygous or homozygous for DJ858. “x” in the genotypes corresponds to which insertion was used (see left most column).
DJ858; VD1 and DJ858; VD2 both display lethality or the crumpled wing phenotype in all females and most males. DJ858; VD1/TM3, Sb and DJ858/CyO; VD1 also display either lethality or crumpled wings.
The self-cross involving VD131 yielded n♂=39, n♀= 41; VD133 n♂= 12, n♀= 8; VD231 n♂= 66, n♀= 82; VD233 n♂= 62, n♀= 66.

<table>
<thead>
<tr>
<th>“x”</th>
<th>Sex</th>
<th>DJ858; VDx</th>
<th>DJ858; VDx/TM3,Sb</th>
<th>DJ858/CyO; VDx</th>
<th>DJ858/CyO; VDx/TM3,Sb</th>
<th>Crumpled Wing</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>%</td>
<td>SD</td>
<td>%</td>
<td>SD</td>
<td>%</td>
</tr>
<tr>
<td>VD131</td>
<td>Male</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>VD133</td>
<td>Male</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>VD231</td>
<td>Male</td>
<td>4.7</td>
<td>2.7</td>
<td>23.9</td>
<td>5.5</td>
<td>22.8</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>0</td>
<td>0</td>
<td>19.5</td>
<td>0</td>
<td>23.2</td>
</tr>
<tr>
<td>VD233</td>
<td>Male</td>
<td>0</td>
<td>0</td>
<td>11.4</td>
<td>3.3</td>
<td>15.8</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>0</td>
<td>0</td>
<td>16.5</td>
<td>5.0</td>
<td>10.6</td>
</tr>
<tr>
<td><strong>Expected proportion of progeny (%)</strong></td>
<td>11</td>
<td>22</td>
<td>22</td>
<td>44</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
Figure 10: Scoring of progeny from crosses between DJ858-GAL4 and UAS-VD flies for the “crumpled wing” phenotype, which was seen in VD121 females, VD124, VD125 females, VD131, VD133, VD134, VD135 females and VD241 at variable frequencies. DJ858 crossed with VD111 yielded n♂=93, n♀=110; VD112 n♂=105, n♀=82; VD113 n♂=94, n♀=92; VD121 n♂=78, n♀=86; VD122 n♂=49, n♀=60; VD124 n♂=99, n♀=118; VD125 n♂=100, n♀=138; VD126 n♂=58, n♀=54; VD131 n♂=81, n♀=88; VD133 n♂=105, n♀=94; VD134 n♂=5, n♀=2 (184 black pupae); VD135 n♂=44, n♀=58; VD211 n♂=95, n♀=94; VD212 n♂=90, n♀=91; VD221 n♂=87, n♀=124; VD231 n♂=103, n♀=131; VD232 n♂=56, n♀=59; VD233 n♂=106, n♀=105; VD234 n♂=44, n♀=53; VD241 n♂=91, n♀=98.
Figure 11: Scoring of progeny from crosses of DJ858; TM3, Sb flies by a subset of VD flies for crumpled wings and stubble bristles. “x” in each genotype indicates that some flies in each set of data carry the TM3, Sb balancer (see legend); all flies are heterozygous for the given UAS-VD insertion. The VD2 strains do not display crumpled wings and the progeny displayed the expected ratios of stubble bristles to wild-type bristles (50:50). Progeny from VD131 and VD133 with stubble bristles had a higher frequency of crumpled wings and made up less than 50% of the total progeny. All progeny that eclosed from the cross with VD134 had wild-type bristles and crumpled wings. DJ858; TM3, Sb/+ crossed with VD131 yielded $n_\text{♂}=123$, $n_\text{♀}=128$; VD133 $n_\text{♂}=100$, $n_\text{♀}=126$; VD134 $n_\text{♂}=1$, $n_\text{♀}=3$ (202 black pupae); VD231 $n_\text{♂}=90$, $n_\text{♀}=123$; VD233 $n_\text{♂}=106$, $n_\text{♀}=107$. 
A CPRG assay performed on 7-9 day old flies obtained from crosses between DJ858 and Bg2, Bg2; 1077 and Bg2; 3.3+1077 showed that one copy of $GAL80$ (1077) did not significantly reduce the expression of $UAS$-$lacZ$ when driven by DJ858 ($p_\delta=0.35$, $p_\varphi=0.55$), while two copies of $GAL80$ (3.3+1077) did so by at least half ($p_\delta=5.0\times10^{-12}$, $p_\varphi=2.6\times10^{-8}$).

**Figure 12:** A CPRG assay performed on 7-9 day old flies obtained from crosses between DJ858 and Bg2, Bg2; 1077 and Bg2; 3.3+1077 showed that one copy of $GAL80$ (1077) did not significantly reduce the expression of $UAS$-$lacZ$ when driven by DJ858 ($p_\delta=0.35$, $p_\varphi=0.55$), while two copies of $GAL80$ (3.3+1077) did so by at least half ($p_\delta=5.0\times10^{-12}$, $p_\varphi=2.6\times10^{-8}$).
**Figure 13:** Scoring of progeny from crosses of DJ858; 3.3+1077 with a subset of UAS-VD strains for the crumpled wing phenotype. 100% of the progeny from VD131, VD133, VD231 or VD233 had wild-type wings. Approximately 15% of males and 30% of females in the progeny of VD134 had wild-type wings. DJ858; 3.3+1077 crossed with VD131 yielded $n_\delta=32$, $n_\varphi=55$; VD133 $n_\delta=66$, $n_\varphi=69$; VD134 $n_\delta=10$, $n_\varphi=10$ (138 black pupae); VD231 $n_\delta=115$, $n_\varphi=119$; VD233 $n_\delta=39$, $n_\varphi=35$. 
CHAPTER 4
DISCUSSION

**UAS-Goα Transgenes are Functional**

Sequencing of pVD2 clones identified a single base-pair mismatch between the clone’s sequence and the expected genomic sequence (Adams et al., 2000). This mutation is located 18bp upstream of the start codon. The concern for a mutation in this location was that it might interfere with the translation of the transcripts. However, the consensus sequence for nucleotides flanking *Drosophila* translation start codons spans from -10 to +5 (Cavener and Ray, 1991). Therefore, the mutation at -18 falls outside of the more highly conserved region, and the -10 to +5 portion of the clone’s sequence matched the consensus sequence (Figure 7). From that information, it was concluded that the -18 mutation should not have an effect on the translation of ORF1 *in vivo*. Once transgenic strains were generated with this construct, this conclusion was found to be correct. VD1 strains must be able to transcribe and translate ORF1 because *UAS-VD1* causes the crumpled wing phenotype when expressed (Figure 10), the same phenotype that was seen with the overexpression of the same ORF in previous experiments (Katanayeva et al., 2010).

When flies are heterozygous for *DJ858-GAL4* and *UAS-VD2*, the wings are able to expand normally – with the exception of VD241, which does display a low frequency of crumpled wings (Figure 10). However, DJ858; VD2 is still lethal, just as is seen with VD1 (Table 2). The crumpled wings in DJ858/+; VD241/+ flies and the lethality of DJ858; VD2 are evidence that *UAS-VD2* is also correctly transcribed and translated.
**Goα Overexpression Phenotypes**

*The Crumpled Wing Phenotype*

Crumpled wings were seen with the expression of *UAS-VD1* in seven out of twelve independent strains (Figure 10). This phenotype has been seen previously with the overexpression of the same ORF encoded by *UAS-VD1*, but with a GAL4 driver that does not mimic the endogenous expression of *Goα47A* (Katanayeva et al., 2010). This phenotype was shown to be caused by the higher levels of the Goα subunit outcompeting the Gsα subunit for a Gβ13F-Gγ1 heterodimer, thereby preventing a Gsα-containing heterotrimer from signalling in the wing maturation pathway.

Since endogenous expression of Goα does not cause the wings to remain crumpled, there seems to be a threshold of Goα expression that needs to be exceeded in order to successfully outcompete Gsα and inhibit wing maturation. In certain VD1 strains, DJ858 could be driving the overexpression of Goα above that threshold. Genes are not expressed at exactly the same level in every individual of the same genotype (Kaern et al., 2005). Goα expression in individuals within a given strain that exhibits crumpled wings likely ranges from below to above the hypothesized threshold. This may explain why some individuals have crumpled wings and some isogenic individuals’ wings are able to mature normally.

The variation in frequencies of the crumpled wing phenotype between strains can be attributed to the fact that transgenes are expressed at different levels depending on their location in the genome (Jiang et al., 1991). Therefore, not only is the phenotype not fully penetrant in a given strain, but each strain may express Goα at different levels, resulting in a variation in penetrance of the phenotype between strains. That means that individuals from strains that do not show the crumpled wing phenotype (VD111, VD112, VD121, VD122, & VD126) likely all
express Goα at a level below the threshold. In the case of VD134, only a small number of flies were able to eclose, despite a successful cross that yielded many pupae, and 100% of flies that did eclose had crumpled wings (Figure 10). This data indicates that DJ858/+; VD134/+ causes pupal lethality in the majority of progeny, likely caused by a very high level of Goα expression by this particular insertion.

The first difference between the two ORFs, and thus the two Goα isoforms, can be seen when flies are heterozygous for DJ858-GAL4 and UAS-VD2. The majority of DJ858/+; VD2/+ flies have wild-type wings and only one of the eight independent VD2 strains, VD241, shows the crumpled wing phenotype that is seen in the majority of VD1 strains (Figure 10).

**Lethality Caused by Overexpression of UAS-VD Transgenes**

When flies are heterozygous for both DJ858-GAL4 and UAS-VD1, a non-fully penetrant crumpled wing phenotype can be seen (Figure 10). For VD131 and VD133, either DJ858-GAL4 or UAS-VD1 being homozygous while the other is heterozygous, or both of them being homozygous at the same time, results in either lethality or the crumpled wing phenotype (Table 2). The zygosity of the third chromosome was identified using the stubble-bristle phenotype, but the same was not possible for the second chromosome because the curly-wing marker could not be scored in flies with crumpled wings. As a result, the exact genotype of those particular flies could not be determined, which makes it difficult to ascertain which genotypes are causing lethality or simply crumpled wings. However, due to the lethality seen when either transgene is homozygous, VD1 flies with crumpled wings are most likely heterozygous for DJ858 and either heterozygous or homozygous for UAS-VD1, depending on the presence of stubble bristles.

When VD2 flies are homozygous for either the driver or the responder and heterozygous
for the other, flies are able to develop and eclose, and have fully expanded wings (Table 2), which is not seen with VD1. However, it is possible for UAS-VD2 expression to cause the crumpled wing phenotype. There is a low number of “DJ858/¿; VD2” flies with crumpled wings, but it cannot be determined whether these flies are heterozygous or homozygous for DJ858. However, the ratios of flies with wild-type wings that were heterozygous for DJ858-GAL4, UAS-VD2, or both, seemed to be as expected. This suggests that the “DJ858/¿; VD2” flies with crumpled wings might actually be a small number of DJ858; VD2 flies that managed to develop and eclose despite the high level of Goα expression, but were not able to expand their wings. This is further supported by the small number of DJ858; VD231 males that were able to eclose with wild-type wings, which was also not seen with either VD1 strain.

The two Goα isoforms differ from each other by 7 of the 21 N-terminal amino acids (the rest of the 354 amino acid-long sequence is identical). It has been shown that the N-terminus of a Gα subunit plays a role in binding to the Gβγ heterodimer (Navon and Fung, 1987). Therefore, it is possible that a difference in the binding affinities of the two Goα isoforms is responsible for the differences in phenotypes seen with the DJ858-driven expression of ORF1 and ORF2. This is consistent with the data shown here since fewer copies of VD1 are needed to cause the crumpled wing phenotype and lethality compared to VD2. Therefore, high levels of the ORF2 isoform of Goα may be sufficient to overcome its lower affinity for Gβ13F-Gγ1 and successfully compete with Gsα in the wing-maturation signalling pathway, accounting for the low frequency of crumpled wings seen in certain VD2 flies.

When DJ858-GAL4 and UAS-VD are heterozygous, seven out of twelve VD1 strains display crumpled wings while only one out of eight VD2 strains does so. The differential affinities of Goα isoforms for Gβγ subunits is a more reasonable explanation than VD2 strains
having overall lower levels of $G_{\alpha}$ expression than VD1 strains. If the latter explanation were correct, the expected outcome for either $DJ858\text{-}GAL4$ or $UAS\text{-}VD2$ becoming homozygous would be a higher frequency of crumpled wings than was seen (Table 2); this frequency would be expected to increase even further when flies become homozygous for both transgenes. Instead, all DJ858/CyO; VD2 and DJ858; VD2/TM3,Sb flies had wild-type wings. DJ858; VD2 is still a lethal genotype, indicating that ORF2 is being expressed at similar levels to VD1, but the two isoforms likely have different roles in signalling. The logical conclusion is that the few amino acid differences are sufficient to result in the different biological activities of the two isoforms.

*Interaction with TM3, Sb Balancer*

Another striking difference between the two ORFs can be seen when looking at the flies with crumpled wings in Table 2. With VD1, the flies with crumpled wings are mostly heterozygous for $UAS\text{-}VD1$. These flies are likely also heterozygous for $DJ858\text{-}GAL4$. With VD2, the flies with crumpled wings are homozygous for $UAS\text{-}VD2$, are likely also homozygous for $DJ858\text{-}GAL4$, and appear at lower frequencies than VD1 flies with crumpled wings do.

However, the frequencies of heterozygous VD1 flies with crumpled wings in this experiment (between 10% and 20%) (Table 2) are much higher than were seen in the experiment with no balancer chromosomes involved (approximately 5%) (Figure 10). The same comparison cannot be made for VD2 since VD231 and VD233 do not cause crumpled wings when they are heterozygous (Table 2, Figure 10). It is difficult to make comparisons between these two experiments, though, because the VD/+ and VD/Tm3, Sb flies developed under different experimental conditions. Therefore, crosses between DJ858; TM3, Sb/+ and five VD strains
were used to investigate whether the presence of the third chromosome balancer was influencing the penetrance of the crumpled wing phenotype and lethality (Figure 11).

When the *UAS-VD1* transgenes were being driven by DJ858 in the presence of TM3, Sb, the penetrance of crumpled wings was higher than it was in the absence of the balancer (Figure 11). In VD131 and VD133 progeny that were heterozygous for *UAS-VD1* but that did not have the balancer, the level of penetrance remained consistent with previous results (Figure 10). However, in flies that carried the balancer as well, the frequency of crumpled wings jumped to approximately 50% in both males and females. In addition, flies that carried the TM3, Sb balancer made up significantly less than the expected 50% of the total number of progeny (Figure 11). This indicates that the TM3, Sb balancer is exacerbating the effect of Goα ORF1 overexpression.

As can be seen in both Figure 10 and Figure 11, the VD134 insertion displays a more severe phenotype than the other VD1 insertions: very few progeny can be obtained from a cross with VD134. Every fly that did eclose, however, had wild-type bristles and crumpled wings (Figure 11). This indicates that the TM3, Sb balancer is lethal in all flies that carry it, which confirms the hypothesis stated above: that TM3, Sb increases the effect of expressing Goα.

As opposed to the results seen with VD1, VD2 progeny (VD231 and VD233) did not show crumpled wings, even in the presence of TM3, Sb (Figure 11). In addition, the frequencies of stubble bristles in the progeny were not significantly different from the expected ratios, with the exception of VD233 females. In this case, TM3, Sb may be responsible for a small amount of VD233 female lethality, indicating that there may be a low level of interaction between Goα ORF2 and TM3, Sb. However, because the lethality is low and only affects one VD2 strain, and because there is still a total absence of crumpled wings in all VD2 flies, the interaction of TM3,
Sb with ORF2 is different from that with ORF1.

The TM3, Sb balancer is a third chromosome that has been rearranged through multiple translocations (Lindsley and Zimm, 1992). It also contains a mutation in the gene Sb that causes “stubble” bristles, which are shorter and thicker than wild-type bristles. As a result, it could be either the Sb\(^{1}\) allele or a translocation-induced mutation that is interacting with Go\(\alpha\) to increase the frequencies phenotypes seen with VD1 strains. One of the break points in TM3 is located in the same cytological position as G\(\beta 76C\) (St. Pierre et al., 2014), indicating that this gene may be mutated on TM3. It is possible that Go\(\alpha\) (ORF1) and G\(\beta 76C\) are part of the same G-protein, in which case, a mutation in G\(\beta 76C\) would reduce the formation of this heterotrimer. That could further increase the amount of “free” Go\(\alpha\) in a cell relative to Gs\(\alpha\), allowing it to more successfully compete for G\(\beta 13F\)-Gy1 and resulting in the increase in penetrance of crumpled wings. The results in Table 2 and Figure 11 indicate that an interaction between TM3 and ORF2 is either non-existent or different from the one with ORF1.

To confirm whether the specific break point at position 76C is responsible for the interaction between ORF1 and TM3, crosses could be performed to generate DJ858/+; VD1/TM3 flies and DJ858/+; VD1/TM6 flies. This would provide a comparison between flies that carry the TM3 balancer and flies that carry a third chromosome balancer with different break points. An experiment could also be performed where a specific mutation in G\(\beta 76C\) could be introduced into DJ858/+; VD1/+ flies to further confirm that this mutation is exacerbating the crumpled wing phenotype and lethality in these flies.

**GAL80 Prevents the Crumpled Wing Phenotype**

GAL80 is a protein that can bind to the activation domain of GAL4 and prevent
transcription of a gene of interest. One copy each of 3.3 and 1077 is able to reduce DJ858-driven transgene expression by about 50% (Figure 12). When GAL80 was introduced into flies that were heterozygous for DJ858 and either VD131 or VD133, the crumpled wing phenotype was completely prevented. With VD134, the insertion that causes pupal lethality when heterozygous, GAL80 was able to reduce the rate of lethality: flies that were able to eclose, as a percentage of total pupae, increased from approximately 2% to 13%. In addition, around 15% of males and 30% of females that eclosed were able to expand their wings into wild-type morphology, which is not seen at all in DJ858/+; VD134/+ flies. This confirms interpretations made earlier, that the crumpled wing phenotype and lethality are a result of above-endogenous levels of Goα expression that vary from insertion to insertion. VD231 and VD233 resulted in progeny with 100% wild-type wings, just as they did without GAL80.

The initial purpose of generating these transgenic strains of D. melanogaster was not to investigate a difference between the two Goα isoforms, but to test their ability to rescue the haloperidol-resistant phenotype of DJ858 mutant flies. Thus, it was important to ensure that the transgenes are being expressed at a level similar to the endogenous expression of Goα47A. In that respect, the presence of crumpled wings, and the subsequent prevention of that phenotype using GAL80, helped ensure that Goα was being kept below the level that causes wing maturation to be inhibited. However, the possibility that the expression is higher than endogenous Goα47A levels cannot be completely excluded.

Significance & Implications for the Future

VD1 and VD2 transgenic strains could be used in a variety of future studies, not only to investigate haloperidol-related phenotypes, but also to distinguish between the two Drosophila
Gox isoforms. If the two isoforms have different affinities for Gβ and Gγ subunits, they would likely compose different heterotrimeric G-proteins and be coupled to different receptors. Studies on Gox in *D. melanogaster* to date have been done using either transgenes corresponding to ORF1 of *Gox47A* (Devambez et al., 2013; Fremion et al., 1999; Katanaev et al., 2005; Katanayeva et al., 2010; Schwabe et al., 2005), or the pertussis toxin (PTX), which inhibits Gox activity (Koon and Budnik, 2012). Studies done with the ORF1 identified receptors that interact with that isoform, such as those that identified Moody, Frizzled, and DmXR. Because studies have not previously been done with ORF2, it cannot be determined whether Moody, Frizzled and DmXR also interact with ORF2. Furthermore, receptors that interact exclusively with ORF2 remain to be identified. Now that transgenic strains exist for both of the ORFs, these questions can finally be addressed.
LITERATURE CITED


Figure A1: Before any experiments were carried out, the haloperidol-resistant phenotype, as identified in the screen, had to be confirmed. The results of the longevity assay performed are presented here: the average lifespan of flies treated with 5mg/ml haloperidol as a percentage of their lifespan when untreated.

Wild-type flies (w^{1118}) live significantly shorter on haloperidol than they do when not treated ($p^\delta=3.3\times10^{-10}$, $p^\varphi=2.0\times10^{-11}$).

DJ858 mutant flies live for a significantly larger proportion of their normal lifespan when on haloperidol, compared to wild-type flies ($p^\delta=7.9\times10^{-5}$, $p^\varphi=1.3\times10^{-7}$).

Sample sizes used: w^{1118} (0mg/ml) $n_\delta=148$, $n_\varphi=144$; w^{1118} (5mg/ml) $n_\delta=166$, $n_\varphi=164$; DJ858 (0mg/ml) $n_\delta=166$, $n_\varphi=164$; DJ868 (5mg/ml) $n_\delta=168$, $n_\varphi=163$. 
Figure B1: Cloning strategy for pVD1.
Figure B2: Cloning strategy for pVD3.
Figure C1: Replicate of CPRG assay. Two copies of GAL80 significantly reduced the expression of the UAS-lacZ compared to in the absence of GAL80 ($p^\beta=6.6\times10^{-22}$, $p^\alpha=7.4\times10^{-17}$).