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Functional Analysis of Corazonin and Its Receptor in *Drosophila melanogaster*

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Functional Analysis of Corazonin and Its Receptor in *Drosophila melanogaster*

A Dissertation Presented for the
Doctor of Philosophy
Degree
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Kai Sha

August 2013

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Dedication

This dissertation is dedicated to my parents, Sha Jian and Liu Li, who have always been there to support me. They encouraged me when I felt the most depressed and embraced my happiness when I succeeded. Without their support and understanding, I could not have made solid progress towards achieving my life goals. I also want to dedicate this dissertation to my grandparents, Sha Binyan, Lin Lin and Liu Yushan, Yang Yinghua, and other members in my family. Finally, I want to dedicate this dissertation to my husband, Zhang Yu, who has provided tremendous encouragement and support to me while completing this work.

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Abstract

Corazonin (Crz) is an amidated undecapeptide originally isolated from the American cockroach. It has been shown to affect diverse physiological functions in a species-specific manner. However, the functionality of Crz in *Drosophila melanogaster* has not yet been determined. To gain insight into the role of Crz signaling in vivo, Crz and CrzR null alleles were obtained by transposable element mobilization. Flies carrying a deficiency uncovering Crz and *pr-set7* loci were generated via P-element excision, and the latter was rescued by *wild-type pr-set7* transgene. A mutation of Crz receptor (CrzR) was generated by *Minos*-element mobilization from *GRHRII*^{MB00583} [*GRHRIIMB00583*] allele. The mutant flies showed normal circadian rhythm and fecundity as compared with the *wild-types*.

Using these mutants, the functions of the Crz signaling system in alcohol metabolism were investigated. Two major enzymes involved in alcohol metabolism are alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH). In this study, Crz expressing neurons and CrzR were identified as important regulators of these enzymes. Flies lacking Crz neurons or CrzR display significantly delayed recovery from ethanol-induced sedation, which is causally associated with fast accumulation of acetaldehyde in the CrzR⁰¹ [*CrzR01*] mutant following ethanol exposure. Consistent with this, Crz and CrzR were found to be required for normal ALDH activity. In addition, CrzR shows a down-regulatory effect on ADH activity, which is transcriptionally

initiated through the protein kinase A (PKA)-dependent pathway. Such transcriptional regulation was found to be exclusive to ADH, but not ALDH mRNA.

To gain the evolutionary aspect of *CrzR*, *CrzR* from the Housefly, *Musca domestica* (*MdCrzR*) was cloned and characterized. *MdCrzR* deduced from the full-length cDNA sequence is a 655-amino acid polypeptide that contains seven *trans*-membrane (TM) domains and other motifs that are characteristics of Class-A G-protein coupled receptors. Although the TMs and loops between the TMs are conserved in other *CrzRs*, *N*-terminal extracellular domain is quite dissimilar. Tissue-specific RT-PCR revealed a high level of *MdCrzR* expression in the larval salivary glands and a moderate level in the CNS. In adults, the expression was broadly observed without significant gender difference, suggesting multifunctionality of the *Crz* signaling system.

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Abbreviations

AC: Adenylate cyclase

Ach: Acetylcholine

ACTH: Adrenocortical Tropic hormone

AKH: Adipokinetic hormone

ADH: Alcohol dehydrogenase

ALDH: Aldehyde dehydrogenase

amn: amnesiac

AP: Alkaline phosphatase

APF: After puparium formation

AUDs: Alcohol use disorders

CA: Corpora allata

cAMP: cyclic Adenosine Mono-phosphate

CAP: Crz associated peptide

CC: Corpora Cardiaca

CNS: Central nervous system

CREB, cAMP response element binding

Crz: Corazonin

Crz-CD: Crz-cell deficiency CrzR: Crz receptor

CrzR-KD: CrzR knockdown
 CYP2E1: Cytochrome P450IIE1
 DD: constant darkness
 DL: Dorsolateral
 DLP: Dorsolateral projection
 DH: Diuretic hormone
 DM: Dorsomedial
 DOC: Deoxycholic acid
 ECD: Extracellular domain
 ELs: Extracellular loops
 ER: endoplasmic reticulum
 GABA: Gamma-aminobutyric acid
 GFP: Green Fluorescent Protein
 GMC: Ganglionic mother cell
 GPCR: G-protein-coupled receptors
 hAT: hobo-Ac-Tam3
 ICD: Intracellular domain
 ILs: Intracellular loops
 JH: Juvenile hormone
 LD: Light and Dark
 MasCrzR : MothCrzR
M. sexta: Manduca sexta
 NAD: Nicotinamide Adenine Dinucleotide
 NB: Neuroblast
 NPF: Neuropeptide F
 NPY: neuropeptide Y

NRE: Negative regulatory element
NRRE: Nuclear receptor response element
NSCs: Neurosecretory cells
PACAP: Pituitary adenylyl cyclase activating peptide
PBS: Phosphate Buffered Saline
PCD: Program cell death
PCR: Polymerase Chain Reaction
PDF: pigment-dispersing factor
PI: Pars intercerebralis
PKA: Protein kinase A
PKAⁱⁿ: PKA inhibitor
PKC: Protein kinase C
PKCⁱⁿ: PKC inhibitor
PL: Pars lateralis
PLC: Phospholipase C
PNs: Projection neurons
PPARs: Peroxisome proliferator-activated receptor
PTG: Prothoracic gland
RT-PCR: Reverse Transcriptase-Polymerase Chain Reaction
SP: signal peptide
TM: *Trans*-membrane
TRITC: Tetramethyl rhodamine isothiocyanate
UTR: Untranslated Region VNC: Ventral nerve cord
vCrz: Crz in the ventral nerve cord
VNC: Ventral Nerve Cord

Chapter 1

Background and significance

I. The neuroendocrine system

Communication between cells is important for the control and coordination of the internal environment of multicellular organisms. It helps the body maintain homeostasis, coordinate behavior, and regulate massive physiological activities. In terms of neuroendocrine system, such communication takes place in two distinct ways; a direct intercellular contact, or long-distance chemical signaling.

The direct communication between neurons develops a high complexity in the nervous system. The synaptic cleft between neurons employs a special chemical communication via small molecule neurotransmitters. A good example would be the neuromuscular junction between a motor neuron and a muscle fiber. Such specialized cell-to-cell contacts involve the release of neurotransmitter from vesicles within the axon terminals of neurons, which then diffuse and bind to the receptors on the dendrite of another neuron, and convey either inhibitory or stimulatory effects on the target neurons.

The second mode of intracellular communication requires the diffusion of chemicals over a long distance. Such communication defines the endocrine system, which involve the release of hormones into the bloodstream, affecting the target cells located far downstream. The blood will eventually circulate and bring the hormone molecules to the target. Although it is indirect way compared to the neuron-neuron interaction, specificity and accuracy are still ensured, since the target cells express very specific and high affinity receptors for a particular hormone.

The third type of intercellular communication is neuroendocrine system in which some produce peptide hormones that are released locally as well as into the blood stream (Thorndyke and Georges, 1986). These neurons are called neurosecretory cells (NSCs). These neuropeptide act on neighboring cells as a neurotransmitter or neuromodulator, or travels through the circulatory system to act on distantly located tissues.

Somata of NSCs are usually located in the central nervous system (CNS), where they receive neuronal input from presynaptic neurons. NSCs project axons to peripheral neurohemal organs and release hormones into the circulatory system. The neurohemal organs store their secretory hormones until they are stimulated by the nervous system. For example, corpora allata (CA), which produces and stores the juvenile hormone (JH), lies just behind the corpora cardiaca (CC). NSCs containing large content of peptide-storing vesicles that distribute throughout the cell body, axon, and synapse (Thorndyke and Georges, 1986). The features of releasing of neuropeptides from the vesicles are quite different from those of the neurons that release only neurotransmitters, since vesicles are not abundant at the synapses. The fusion of vesicles with the cell membrane not only occurs at the axon terminals but also along the soma and axon. Thus, these NSCs affect multiple cells that are within reach of the projections.

In insects, the neurosecretory system consists of several sets of NSCs that locate in the CNS. The majority of NSCs are found in the dorso-medial pars intercerebralis (PI) and dorso-lateral pars lateralis (PL) part of the brain (Pipa, 1978; Siegmund and Korge, 2001; Veelaert et al., 1998). These NSCs project their axons toward a set of neurohemal organs, such as the CC and CA. In *D. melanogaster*, two major neurohemal organs, CC, CA, and the prothoracic gland (PTG), fuse together into a single complex called the ring gland. Through the releasing sites on CC and CA, hormones produced by NSCs can either be released into the circulatory system, or act locally on the glandular cells of these neurohemal organs and control the release of hormones from CC and CA. The anterior tip of the dorsal vessel is surrounded by the ring gland, which benefits the releasing of hormones into the circulatory system.

II. Neuropeptides

Neuropeptides are small bioactive peptides produced by specialized neurons and NSCs and are involve in a wide range of physiological processes, including homeostatic metabolism, behavior,

reproduction, and neuronal signal transduction pathways (Li et al., 1999; Li et al., 2003; Nassel, 2002; Pohorecky, 1977; Sandman et al., 1999). Depending on the releasing site and the target tissue, neuropeptides may act as neurotransmitters, hormones or neuromodulators. For instance, the pituitary adrenocortical tropic hormone (ACTH), in addition to its effects on central and peripheral neuronal functions, is also released to the adrenal cortex and stimulates the production of corticosteroids. ACTH has also been shown to have an effect on the immune system that is closely related to stress and disease. Neuropeptides are also frequently found to co-exist with the classical neurotransmitters such as acetylcholine (ACh), norepinephrine and gamma-aminobutyric acid (GABA) (Hokfelt, 1991).

The distinction of neuropeptides from small molecule neurotransmitters, such as dopamine, is that they are much larger and possess multiple recognition sites for their specific receptors. This provides higher affinity of ligand receptor binding of neuropeptides, which makes it capable of fulfilling biological effects with lower quantities.

The transcription of neuropeptides coding genes is regulated in a cell-specific manner, which leads to the expression of neuropeptides in a limited set of neurons in the nervous system (Benveniste and Taghert, 1999; Taghert, 1999). Most of the neuropeptides are encoded with genes that initially produce large precursor molecules. These precursors enter ER and then follow the secretory pathway, during which they undergo further processing via several enzymatic reactions, including cleavage by prohormone convertase (Strand, 1999). After cleavage the peptides can undergo posttranslational modifications such as amidation of carboxy groups, disulphide bond formation, sulfation of tyrosyl groups and pyroglutamate formation in the amino terminus (Strand, 1999; Zupanc, 1996). The mature neuropeptides are packed in large vesicles and transported to the releasing sites (Karhunen et al., 2001; Zupanc, 1996). The releasing of neuropeptides occurs upon the depolarization of the neurons (Zupanc, 1996). It happens along the axon projections, through either typical synaptic or non-synaptic sites.

Strong evolutionary links can be found between members of the same peptide family, whose structures and functions are usually conserved throughout evolution. A good example would be the neuropeptide Y (NPY), which is involved in food intake and energy balance (Yang et al., 2009). NPYs among different mammalian species exhibit strong conservation, with a 92% sequence similarity between mouse and human (Blomqvist et al., 1992). *Drosophila* Neuropeptide F (NPF), which is homologous to mammalian NPY, is also showing a remarkable degree of conservation during evolution (Brown et al., 1999; Garczynski et al., 2002; Wen et al., 2005).

III. Neuropeptide receptors

The receptors of neuropeptides are usually seven-transmembrane G-protein-coupled receptors (GPCR) (Broeck, 2001; Hewes and Taghert, 2001; Strand, 1999; Zupanc, 1996). When a hormone or neuropeptide binds to the associated GPCR, GPCR undergoes a conformational change that favors the coupling with specific G-proteins. G-proteins are heterotrimers that consist of G_{α} , G_{β} and G_{γ} . The binding of a ligand to the associated GPCR triggers an allosteric change in the receptor, leading to the exchange of GDP for GTP on G_{α} . The activated G_{α} dissociates from both the receptor and $G_{\beta\gamma}$, which then interact with adenylate cyclase (AC) to regulate the synthesis of cyclic-adenosine monophosphate (cAMP), or phospholipase C (PLC) to control intracellular calcium levels.

In insects, only few receptors have known to bind specific neuropeptide ligands. Some of them share structural and/or functional features in an evolutionary aspect. These include the *Drosophila* NPF receptor, which is related to the vertebrate NPY and snail NPY/NPF receptors (Garczynski et al., 2002). The adipokinetic hormone (AKH) receptors are shown to share structural similarities to gonadotropin-releasing hormone receptors (Staubli et al., 2002). The *Manduca* diuretic hormone (Giammattei et al.) receptor, functioning on the stimulation of AC, has been shown to share structural similarity with the calcitonin/secretin receptor family of mammals. In *Drosophila*, there

are around 40 putative neuropeptide receptors in the genome (Brody and Cravchik, 2000; Broeck, 2001; Hewes and Taghert, 2001).

Multiple ligands bind to the receptors on their unique binding sites; such interactions stabilize different ligand-GPCR conformations, coupling selective G proteins to activate distinct downstream signaling pathways (Perez and Karnik, 2005; Seifert and Dove, 2009). There are numerous members in the G protein family, including 16 G_{α} subunits, 5 G_{β} subunits, and 14 G_{γ} subunits (Milligan and Kostenis, 2006). The selective pattern of G proteins that is activated by a given GPCR-ligand interaction determines the downstream signaling cascades. Recent studies have shown such a functional selectivity of several GPCRs, including β -adrenoceptors, opioid, tachykinin, serotonin and dopamine receptors (see reviews (Kobilka, 2007; Perez and Karnik, 2005; Pineyro and Archer-Lahlou, 2007), in which different ligands stabilize distinct receptor conformations. These ligand-induced GPCR conformations have variable affinity for one G protein over another, determining the downstream signaling. A good example is the cannabinoid 2 receptor (Shoemaker et al., 2005). When CHO cells expressing this receptor are treated with three different agonists, distinct effectors are activated, including AC activity, mitogen-activated protein kinase activity, and calcium transients.

IV. Corazonin signaling

IV-1. Corazonin

Corazonin (Crz) is an amidated undecapeptide that was originally isolated from the American cockroach, *Periplaneta Americana* (Veenstra, 1989). Later on, similar or identical peptides were discovered from several other insect species, including Orthoptera (grasshopper and crickets) (Hua et al., 2000), Lepidoptera (moths), Hymenoptera (bees and ants) and Diptera (flies and mosquitoes), forming a neuropeptide family (Nassel, 2002).

Similar to other neuropeptides, Crz is also released from a larger precursor and cleaved via posttranslational processing. The precursor of Crz consists of a signal peptide (SP) and mature Crz peptide, followed by Crz associated peptide (CAP). A posttranslational processing site GKR is present between a mature peptide and CAP. The mature peptide contains 11 amino acid residues. Two major members of the Crz family are [Arg⁷]- and [His⁷]-Crz, while other variants are identified from restricted groups of insects: [Thr⁴, His⁷]-Crz in the honey bee (Roller et al., 2006), [Gln¹⁰]-Crz in the American crane fly, [His⁴, Gln⁷]-Crz in the members of the Order Mantophasmatodea and [Tyr³, Gln⁷, Gln¹⁰]-Crz in the bumble bee (Predel et al., 2007). Surprisingly, multiple lines of evidence indicate the lack of Crz and its cognate receptors in several beetle species (Order Coleoptera) and aphid (Hauser et al., 2008; Huybrechts et al., 2010; Li et al., 2008; Roller et al., 2003; Weaver and Audsley, 2008).

Crz in most of the *Drosophila* members of the Sophophora subgenus are characterized as single-intronic gene (see the Flybase for a brief phylogenetic tree: flybase.org/blast/). Double-intronic structure has been observed from *Drosophila virilis*, *Musca domestica*, *Apis mellifera*, and two mosquito (*Anopheles gambiae* and *Aedes aegypti*) Crz genes, with one intron in the 5'-UTR and the other one in the ORF (Sha et al., 2012).

Spatial distribution of Crz-producing neurons is conserved within the CNS of insects (Cantera et al., 1994; Choi et al., 2005; Hansen et al., 2001; Roller et al., 2006; Roller et al., 2003; Veenstra and Davis, 1993; Verleyen et al., 2006). The primary expression site in the brain is a group of NSCs in the pars lateralis. In addition to this site, species-specific expression sites have also been found. Notably, Crz mRNA expression in numerous cells in the optic lobe medulla was observed only in *D. melanogaster* (Choi et al., 2005; Lee et al., 2008).

Although the sequence and structure of Crz is evolutionarily conserved among different insect species, it has been shown to affect diverse physiological functions in a species-specific manner. Unlike the effect on cardio-acceleration in the cockroach (Veenstra, 1989), Crz plays a role in the induction of dark cuticular pigmentation in the migratory locust, *Locusta migratoria* (Tawfik et al.,

1999). Crz also has an effect on the developmental processes, such as reducing the spinning rate of the silkworm, *Bombyx mori* (Tanaka et al., 2002), and inducing ecdysis behavior in the moth, *Manduca sexta* (Kim et al., 2004b). Recently, it has also been reported to promote the transferring of sperm, and reduce the copulation duration in the fruit fly, *D. melanogaster* (Tayler et al., 2012).

IV-2. *Drosophila* Corazonin

Previously, Crz coding gene has been cloned and characterized in *D. melanogaster*. The Crz precursor in *D. melanogaster* consists of three distinct regions: 19 amino acids signal peptide, 11 amino acids Crz, and 121 amino CAP (Fig. 1-1).

Crz neurons are specialized neurons derived from neuroblast (NB) 7-3 (Fig. 1-2). NB7-3 gives rise to six cells through several symmetric and asymmetric divisions. The first asymmetric division of NB7-3 yields a regenerated neuroblast and a ganglionic mother cell (GMC). This GMC undergoes mitotic division which gives rise to two daughter cells, EW1 and GW. The EW1 cell later on develops into a serotonergic interneuron, while the GW cell develops into a motoneuron. The regenerated neuroblast undergoes another asymmetric division and produces a new NB7-3 and a GMC. The GMC then divides into a EW2 cell which later on becomes another serotonergic interneuron, while the other daughter cell undergoes apoptosis. The new NB7-3 then produces GMC that divides into a EW3 and a daughter cell. While the daughter cell is removed via program cell death (PCD), the EW3 cell finally develops into Crz neuron (Karcavich and Doe, 2005; Lundell et al., 2003; Novotny et al., 2002).

Crz peptides are expressed in a total of 24 neurons. During the larva stage, Crz neuropeptides are detected in a group of three pairs of dorsolateral (DL) neurons and one pair of dorsomedial (DM) neuron in the brain region, and eight bilateral pairs of Crz neurons in the ventral nerve cord (VNC) in an orderly fashion (Choi et al., 2005; Lee et al., 2008).



Figure 1-1. Crz precursor in *D. melanogaster*. The precursor contains a signal peptide (SP) and Crz, followed by Crz-associated peptide (CAP). A posttranslational processing site (GKR) is present between Crz and CAP. Crz is released from a larger precursor via posttranslational processing.

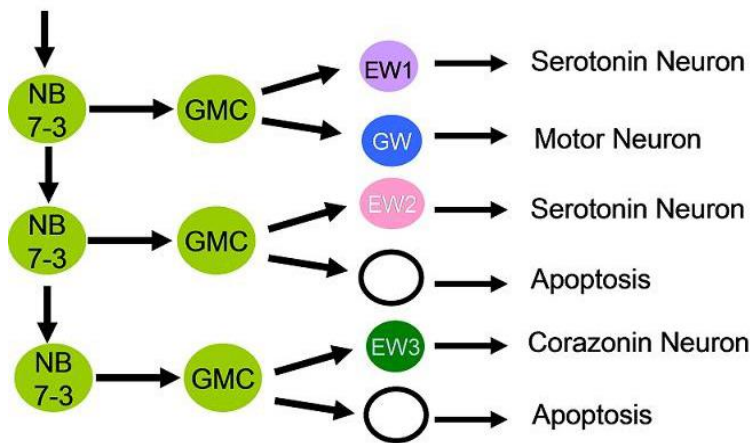


Figure 1-2. Development of NB7-3 lineage. Three contralaterally projecting interneurons (EW1, EW2, and EW3) and one motor neuron (GW) are derived from NB7-3. (NB, neuroblast; GMC, ganglionic mother cell)

During metamorphosis, *Crz* expression changes substantially. Three to four adult-specific *Crz* neurons are added to the region where DL neurons are, and form a cluster of neurons that are called dorsolateral projection (DLP) neurons (Choi et al., 2005; Lee et al., 2008). None of the v*Crz* neurons are persistently present as they undergo PCD within 6 hours after puparium formation (APF) (Choi et al., 2006; Lee et al., 2008; Lee et al., 2011). These observations suggest that v*Crz* neurons have larva-specific functions. Interestingly, there are another four adult *Crz* neurons in the abdominal ganglion, which are male-specific (Lee et al., 2008). Thus the expression of *Crz* is cell specific, developmental stage specific and also gender-specific.

In the house fly, there are a group of three *Crz* neurons in the DL protocerebrum, and eight pairs of bi-lateral neurons in VNC in the larvae. In adults, the expression was found exclusively in a cluster of five to seven neurons per brain lobe (Sha et al., 2012).

In the adult fruit flies, *Crz* is typically produced by a group of NSCs in the PL of the protocerebrum, which send their projections into the CC of ring gland (Choi et al., 2005). As a neurohormone and neuromodulator, *Crz* is predicted to deliver multiple biological functions (Lee et al., 2008). Using *Crz*-cell specific ablation, these neurons were shown to be involved in the regulation of trehalose (Lee et al., 2008) levels as well as stress-induced behavior and dopamine production (Zhao et al., 2010) in the fruit flies.

Previous studies using targeted cell ablation indicated its role on the *Crz* signaling pathway in a series of biological processes. Targeted cell ablation was accomplished by inducing transgenic expression of one of the apoptotic genes (*hid*, *grim* or *reaper*) (e.g., Lee et al., 2008). As a result, flies lacking *Crz* neurons (*Crz*-cell deficiency; for short, *Crz*-CD) showed no difference from controls with respect to the sensitivity to ethanol vapor derived from 30, 50 and 70% of ethanol (Choi, 2009). However, the *Crz*-CD flies showed significantly delayed recovery from ethanol sedation. Also, these flies were insensitive to odor perception. To extend the study on *Crz* signaling pathway, *Crz* receptor (*CrzR*) was specifically knocked down (*CrzR*-KD) via *CrzR* RNAi expression, driven by a

universal driver *actin-gal4*. *CrzR*-KD also led to a defect in odor perception (Choi, 2009). To further determine whether the absence of *Crz* production causes the hangover phenotype and odorant insensitivity, we attempted to generate *Crz* and *CrzR* null mutants via transposable element mobilization for further analysis.

IV-3. Corazonin receptor

A member of the GPCR encoded by *Drosophila* gene *CG10698* has been identified as the receptor for *Crz* neuropeptide (Cazzamali et al., 2002; Johnson et al., 2003; Park et al., 2002a). Its ortholog in the moth also exhibits a high sensitivity and selectivity for *Crz*, as injection of *Crz* initiates the ecdysis behavioral sequence (Kim et al., 2004a). Later on, other homologous receptors have been predicted in several genome databases (Veenstra, 2009). *Drosophila* *CrzR* is also closely related to the gonadotropin-releasing hormone (GnRH) receptors (Hansen et al., 2010; Park et al., 2002b; Roch et al., 2011)

CrzR is a member of Class-A GPCR (Sha et al., 2012). The best known receptors in this class are the rhodopsin-like receptors. The class-A GPCR members share several structural features (Hofmann et al., 2009; Rosenbaum et al., 2009). These include the E/DRY motif that is involved in agonist binding, receptor structure and activation process (Fraser et al., 1988), the CWxP motif serving as a rotamer toggle switch (Hofmann et al., 2009; Shi et al., 2002), and the NPxxY motif (Fritze et al., 2003) that is important for the formation of the TM1-TM2-TM7 network (Hofmann et al., 2009). Disulfide bond that links the first and second extracellular loops (ELs) is also a feature shared in all Class-A GPCRs (Hausdorff et al., 1990). These conserved motives were also found in *Drosophila* *CrzR*, as well as in other insect species including *Musca domestica* (Sha et al., 2012). In some GPCRs, palmitoylated Cys residues in the cytoplasmic tail anchor the receptor to the plasma membrane, forming extra cytoplasmic loops (Chini and Parenti, 2009).

V. Alcohol induced behavior and metabolism

V-1. Alcohol related behavior and AUDs

Alcohol is one of the most consumed beverages and abused drugs in the world. Acute alcohol consumption leads to short-term behavioral changes. For example, low doses of alcohol consumption result in stimulation, while high dose leads to depressant effects (Babor et al., 1983a; Pohorecky, 1977). In addition, chronic alcohol consumption can cause alcohol use disorders (Weaver and Audsley) (Weaver and Audsley), including alcohol abuse and alcohol dependence. Such uncontrolled alcohol consumption leads to serious health risks, such as liver cirrhosis and various types of cancer (for a review, see (Bagnardi et al., 2001)), which makes it important to study the factors that contribute to the high consumption of alcohol.

Several studies revealed the relationship between acute ethanol sensitivity and the risk to develop an AUD. In general, increased sensitivity to stimulant effects (excite the body), is correlated with a higher risk for developing AUDs; while increased sensitivity to depressant effects (sedative effects), is associated with a lower risk for developing AUDs (Morean and Corbin, 2010). Thus, measuring of the sensitivity to acute alcohol exposure can provide important information for the risk to develop AUDs.

As for the difficulty and cost to study ethanol sensitivity in humans, behavioral response to alcohol has been studied in several animal models, including fruit flies, *D. melanogaster* (Guarnieri and Heberlein, 2003). Fruit flies share numerous similarities with mammals in alcohol related biological processes, at behavioral as well as cellular and developmental levels. The fruit flies exhibit similar responses to ethanol as compared to those of mammals (Bellen, 1998; Heberlein, 2000; Scholz et al., 2000; Wolf and Heberlein, 2003). Low doses of ethanol exposure stimulate their locomotion; while high doses may gradually decrease the locomotion and finally render them intoxicated or sedated. In addition, the dosage dependent responses to ethanol of flies indicates that they develop ethanol tolerance with chronic ethanol treatment, which may be the result of brain adaptation (Scholz et al.,

2000). Also, various genetic and transgenic tools and extensive bioinformatics available for *D. melanogaster* make this species an attractive model system to investigate genetic influence on alcohol related behavior.

Acute ethanol sensitivity on *Drosophila* has been studied for decades. Different systems/methods have been developed for this study. The inebriometer, the first apparatus used for testing the ethanol sensitivity (Pohorecky, 1977), contains a vertical column with mesh baffles. When flies are placed on the top of the column, which had been saturated with ethanol vapor, they remain at the top in the beginning but then lose postural control, and will fall from one baffle to the next. The average time needed for the flies to reach the bottom was recorded, which indicated the sensitivity of a particular fly strain to ethanol. Recovery from ethanol intoxication is another method to test the sensitivity (Berger et al., 2004; Cowmeadow et al., 2005). Flies subjected to constant ethanol vapor for a certain time period in a locomotion tube are transferred into ethanol-free environment. The speed of recovery is considered an indicator of the sensitivity to ethanol.

Genetic studies have reported several genes and signaling pathways that regulate the acute response to ethanol. These genes are evolutionarily conserved in mammals as well as in *Drosophila*, include Protein kinase A (PKA), Protein kinase C (PKC) and large-conductance calcium-activated potassium channels (BK channels) (Babor et al.). Neuropeptides have also shown to be involved in the regulation of ethanol sensitivity in *Drosophila*. Flies deficient in NPF signaling showed decreased alcohol sensitivity, whereas those overexpressing NPF exhibited the opposite phenotype (Wen et al., 2005). *Drosophila* memory gene *amnesiac* (*amn*), encoding a neuropeptide homologous to mammalian pituitary adenylyl cyclase activating peptide (PACAP), is required for reducing the sensitivity to alcohol-induced loss of postural control (Moore et al., 1998). Knocking down of *arouser* in the pigment-dispersing factor (PDF) expressing circadian pacemaker neurons is correlated with increased alcohol sensitivity (Eddison et al., 2011).

V-2. Alcohol metabolism

In mammals, detoxification of ethanol is mainly occurred in the livers, in which ethanol is first oxidized to acetaldehyde by alcohol dehydrogenase (ADH), and then further oxidized into acetate by mitochondrial aldehyde dehydrogenase (ALDH). Acetate is then converted into acetyl-CoA and enters the tricarboxylic acid cycle. Another enzyme in the liver is called cytochrome P450IIE1 (CYP2E1), which catalyzes alcohol into acetaldehyde. The expression of CYP2E1 is inducible by ethanol, with increased protein stability at low levels of ethanol and increased transcription at high levels of ethanol (Lieber, 1999).

The fruit flies feed on fermenting fruits which give rises to ethanol as a byproduct. They use ethanol as an energy source as well as the substrate for lipid synthesis. The metabolic system in *Drosophila* is conserved with the ones from the mammalian species. *Drosophila* ALDH has been found to be the homolog of mammalian ALDH-2. *Drosophila* ADH belongs to the short-chain dehydrogenase/reductase family (SDR), which is structurally different from those of the vertebrate ADH.

The intermediate product of ethanol metabolism is acetaldehyde, which causes adduct formation, leading to the dysfunction of various key proteins and DNA damage (Setshedi et al., 2010). It has also been suggested as a carcinogen, according to the International Agency for Research on Cancer (IARC) (1999). Thus, accumulation of acetaldehyde introduces significant toxic effects during alcohol ingestion. The efficient removal of acetaldehyde, which requires well functional ethanol metabolism enzymes, is important for the survival of the living organism.

Genetic factors have been shown to affect the activity of alcohol metabolic enzymes. Both ADH and ALDH exhibit genetic polymorphisms with ethnic distributions. Such polymorphisms result in enzymes with different kinetic properties. For example, certain ADH1B and ADH1C variants encode highly active ADH enzymes, leading to more rapid conversion of alcohol to acetaldehyde. These variants mostly exist among the Caucasians (Edenberg, 2007). A variant of the ALDH2 gene

encodes an inactive ALDH enzyme, resulting in rapid acetaldehyde accumulation during alcohol consumption. This variant causes a syndrome known as acute alcohol sensitivity, which mostly exists among the eastern Asians (Alcorta, 1991; Impraim et al., 1982; Peng et al., 1999; Yoshida et al., 1984). Polymorphism of ADH was also found in *Drosophila*. Two major alleles, *Adh^F* and *Adh^S* (F, fast; S, slow), exist in the natural population. *Adh^F* encodes an ADH enzyme with higher activity than the one encoded by *Adh^S* (van Delden et al., 1978). Flies bearing the *Adh^F* allele are more resistant to ethanol than the ones with the *Adh^S* allele (Briscoe et al., 1975; Cavener, 1979).

The regulation of ADH/ALDH activity also happens at the transcriptional level. Peroxisome proliferator-activated receptor (PPARs), which is a transcription factor, has been found to bind to the ALDH nuclear receptor response element (NRRE) *in vitro*. However, it has no *in vivo* effect on the transcription of *Aldh* (Crabb et al., 2001). Another transcription factor, the aromatic hydrocarbon receptor, was found to mediate a positive control of mouse *Aldh-1* (Nebert et al., 1990). Two cis-acting elements, GATA-2 and HNF-3beta, have been shown to contribute to the regulation and tissue specificity of human *Adh1A* (Dannenberg et al., 2005). A negative regulatory element (NRE) of *Adh-1* has been shown to down-regulate its transcription (Yu et al., 1994). Post-translational modifications also show effects. For instance, upon oxidative or nitrosative stress, the critical Cys residues of ALDH2 and its isozymes undergo oxidative modifications that likely inhibit its activity (Song et al., 2011).

Pharmacological approaches revealed the effects of hormones, such as thyroid hormones (Hillbom and Pikkarainen, 1970; Israel et al., 1975; Ylikahri and Maenpaa, 1968) and growth hormone (Mezey and Potter, 1979; Potter et al., 1989) on liver ADH activity. Stress that stimulates the hypothalamo-hypophyseal-adrenocortical axis and the sympathetic nerve system can enhance ADH activity (Mezey et al., 1979).

Dietary alcohol and carbohydrates also influence *Drosophila* ADH activity (Fry et al., 2004; Mckechnie and Geer, 1984). Larvae reared on media with 2.5% ethanol developed induced ADH activity of at least 2.5-fold higher than the activity of larvae fed with non-ethanol media. The

increased ADH activity is because of the combined effects from increased concentrations of the isozymes ADH-3 and ADH-5 but decreased concentration of ADH-1 in larvae. Sucrose showed a similar but lesser effect on the activity of ADH.

However, there are no defined endogenous pathways that regulate the activities of ADH and ALDH. Here, we investigated the biological functions of the *Crz* signaling. Using genetic and transgenic tools, we found that flies devoid of *Crz* neurons displayed significantly delayed recovery from ethanol-induced sedation. Such 'hangover' phenotype was also found in *CrzR*-null mutant we generated via transposable element mobilization. In association with this phenotype, we also observed faster acetaldehyde accumulation in *CrzR*-null mutant flies during ethanol sedation. Such acetaldehyde accumulation is probably due to higher alcohol dehydrogenase activity and lower ALDH activity in the mutant. In addition, *CrzR* shows a down-regulatory effect on ADH transcription level through the PKA-dependent pathway. Such up- and down- regulation of ALDH and ADH, respectively, activated by *CrzR*, efficiently prevent the acetaldehyde from accumulating.

VI. Anatomical features of the olfactory system

In animals, the olfactory cues are often used to provide location of the food source, to attract potential mates and to explore the environment. The olfactory systems of insect and mammals share various similarities. In *Drosophila*, each of the olfactory sensillum is loaded with up to four olfactory receptor neurons. Olfactory signaling starts by the binding of small odor molecules on the dendrites of the odorant receptors (ORs) in these neurons.

The molecular identities of the ORs have been well studied in the fruit fly, with a total of 45 ORs expressed in the adult olfactory neurons (Couto et al., 2005). These ORs are characterized as seven trans-membrane receptors with inverted topology as compared to the GPCRs (Benton et al., 2006). A large number of ORs is not randomly expressed in individual olfactory receptor neurons (ORNs).

One of the ORs, Or83b, is expressed in most of the ORNs (Larsson et al., 2004). Each ORN expresses selective ORs, usually Or83b plus one to three other receptors (Couto et al., 2005; Fishilevich and Vosshall, 2005), determining the pattern of olfactory input.

In *Drosophila*, there are around 1300 ORNs in the antenna (Fig. 1-3). These neurons project their axons bilaterally into the antennal lobes, where they terminate at around 50 glomeruli (Larsson et al., 2004; Neuhaus et al., 2005). ORNs that express the same type of ORs, arborize their axon terminals in the same glomerulus (Vosshall et al., 2000).

The ORNs are characterized by about 50 classes, depending on the combinations of ORs expressed. Thus, there are also about 50 glomeruli receiving olfactory input from each class of the ORNs (Laissue et al., 1999).

In the antennal lobe, there are two broad types of neurons, projection neurons (PNs) and local neurons (LNs). About 150 PNs and 100 LNs exist in each antennal lobe. The olfactory signal from peripheral sensory organs transfers to the antennal lobe, and is relayed by PNs to higher processing centers in the lateral horn (LH) and the mushroom body (MB) (Wilson and Laurent, 2005). Most of these PNs are cholinergic excitatory neurons, while some are GABAergic inhibitory neurons (Jefferis et al., 2007; Okada et al., 2009; Wilson and Laurent, 2005). They send axons in a bundle through the inner antennocerebral tract, while only a few PNs sending axons through the middle antennocerebral tract (Lai et al., 2008; Marin et al., 2002). Although PNs send input into the higher processing centers, there is, so far, no evidence showing the feedback from the LH or the MB. However, the antennal lobe is also controlled by the neuromodulatory neurons that release neurotransmitters, including dopamine, octopamine and serotonin (Nassel, 2002).

The LNs, unlike the PNs, are primarily GABAergic inhibitory neurons, and do not form connections outside the antennal lobe (Hoskins et al., 1986; Malun, 1991). There are also a few LNs that release Ach, which are excitatory (Shang et al., 2007). Both excitatory and inhibitory LNs connect each glomerulus with other glomeruli and modulate the activities of PNs. The excitatory LNs are known to be sufficient to cause spiking responses in the PNs, where no direct input is

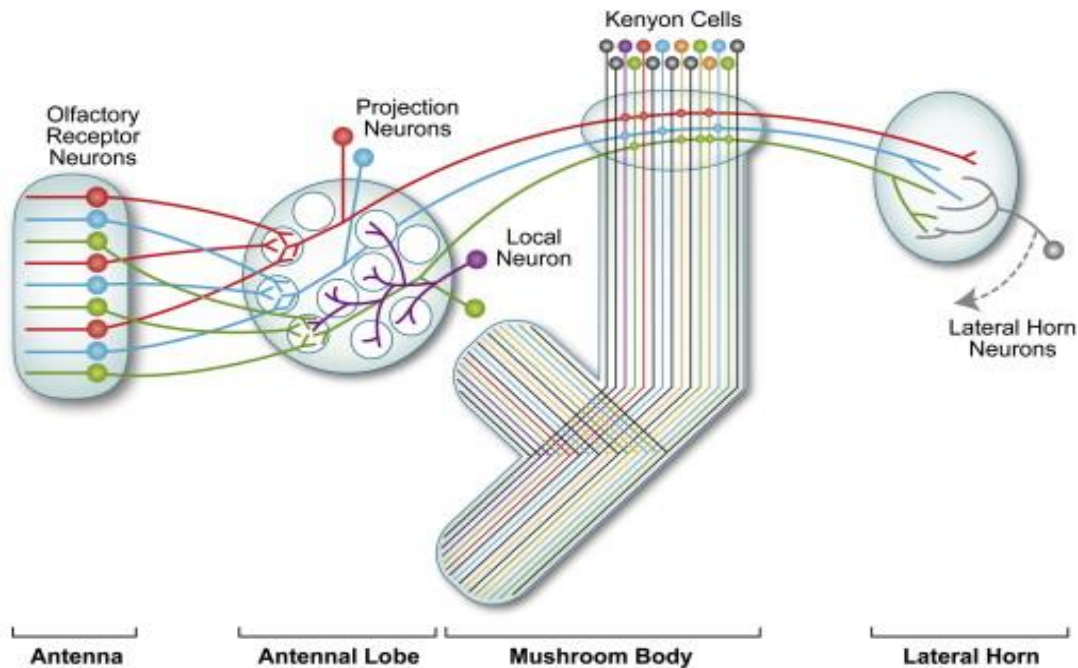


Figure 1-3. Schematic illustration of the glomerular organization of the *Drosophila* antennal lobe. The axons of neurons expressing the same type of odorant receptor targeted on the same glomeruli in the antennal lobe. Different types of olfactory receptor neurons that express a specific combination of odorant receptors are represented by blue, green and red. Each excitatory PN sends its dendrites to a single glomerulus, where it receives extensive input from a single class of olfactory receptor neurons (in same color), and sends an axon to a higher brain center (purple). The inhibitory LNs (magenta) project to multiple glomeruli. PNs then project to the LH neuropile where they contact LH neurons. On their way to the LH, PNs send axonal projections to the calyx of the mushroom body where they form synapse terminals with Kenyon cells. Figure adopted from Sebastian and Gregory, 2008.

received from the ORNs (Olsen et al., 2007; Shang et al., 2007). The inhibitory effects of LNs are well studied, and most of such modulations are known to occur at the terminals of ORNs (Jefferis et al., 2007; Wilson and Laurent, 2005). There are feedback inputs as they also receive signals from both ORNs and PNs (Wilson and Laurent, 2005).

Using physiological and behavioral analysis, genetic effects on olfaction have been studied in invertebrates for the past twenty years (Hallem et al., 2006; Vosshall and Stocker, 2007). The olfactory system consists of many interacting genes, which also show combined effect with the environment. A complex genetic architecture has been revealed to shape animals' olfactory behavior, (Anholt et al., 2003; Anholt et al., 2001; Anholt et al., 1996; Sambandan et al., 2006).

Chapter 2

Loss of function mutation of *Crz* and *CrzR*

Part of this study has been submitted for publication.

I. Abstract

Crz is an amidated undecapeptide originally isolated from the CC of the American cockroach. It has been shown to affect diverse physiological functions in a species-specific manner. In *D. melanogaster*, however, the functionality of *Crz* has not yet been determined. To gain insight into *Crz* signaling, *Crz* and *CrzR* null alleles were obtained by transposable element mobilization. Flies carrying a deficiency on both *Crz* and *pr-set7* genes were generated via *P*-element excision. Since homozygous *pr-set7* is pre-pupal lethal, a construct containing full length of *Crz* and *pr-set7* genes was introduced into the mutant background. Two premature termination codons was introduced in the immediate upstream of the mature *Crz* peptide. Immunochemistry confirmed the absence of *Crz* peptide expression from *Crz null* allele, while *Crz* mRNA expression confirmed by *in situ* hybridization. Transgenic expression of *pr-set7* under *actin-gal4* also rescued the pre-pupal lethality. To further understand the signaling pathway of *Crz*, a complete loss of function mutation of its receptor (*CrzR*) was generated by *Minos*-element mobilization from *GRHRII*^{MB00583} allele. RT-PCR confirmed the absence of *CrzR* mRNA expression from *CrzR null* allele. The mutant flies showed normal circadian rhythm and fecundity as compared with the *wild-types*.

II. Introduction

Several studies using *Crz*-cell specific ablation suggest the involvement of *Crz* signaling in the regulation of trehalose (Lee et al., 2008) levels, as well as stress-induced behavior and dopamine production (Zhao et al., 2010) in the fruit flies. However, *Crz* or *CrzR null* mutations are required to gain definitive evidence for these biological functions of *Crz* signaling. In *Drosophila*, complete loss of function alleles can be generated via numerous transposable elements mobilization.

The activities of transposable elements are one of the major sources for mutant generation. Due to their replicative ability or selection, transposable elements have been maintained in the genome in evolution (Charlesworth et al., 1994; Finnegan, 1989). There are several classes of transposable elements. One of them, named Class II elements, is found in eukaryotes, and characterized as the inverted repeats at 3' and 5' terminals (Finnegan, 1989). These Class II elements mobilize via a cut-and-paste mechanism (Kaufman and Rio, 1992).

There are three main families in the Class II elements, including the *P*-family (*P*-element from *D. melanogaster*), the *hAT* (*hobo-Ac-Tam3*) family (*hobo* from *D. melanogaster*, *Ac* from maize, and *Tam3* from the snapdragon, *Antirrhinum majus*) and the *Tcl-mariner* family (*Tcl* from *Caenorhabditis elegans* and *mariner* from *D. mauritiana*) (Calvi et al., 1991; Engels, 1996; Henikoff and Henikoff, 1992).

The autonomous *P*-element is around 2.9-kb (available from Genbank). They are characterized by perfect 31-bp terminal inverted repeats and an 11-bp subterminal inverted repeats (Ohare and Rubin, 1983). These repeats usually flank a transposase gene composed of four exons. The nonautonomous *P*-elements occur naturally through internal deletions of the transposase gene. The structures still retain the parts of the sequence required in *cis* for transposition. Mobilization of the nonautonomous *P*-element requires exogenous supply of the transposase. Many artificial nonautonomous *P*-elements were designed to replace the transposase gene by another gene of interest, often a marker or reporter.

Minos-element belongs to the *Tcl-mariner* family. It was first found in *Drosophila hydei*, with a size of around 1.8-kb (Franz and Savakis, 1991). Including 255-bp inverted repeats at the terminals (Franz et al., 1994; Franz and Savakis, 1991). *Minos*-element is nonautonomous, which requires integration of *Minos* based transposases into germ line chromosomes (Loukeris et al., 1995).

Mobilization of the class II transposable elements results in double-strand breaks in the genome. DNA repair can be conducted by two alternative mechanisms in different systems. In plants, the broken ends induced by *Ac* and *Tam* can be repaired through ligation (Saedler and Nevers, 1985). The second way is gap repair, in which the excision sites are conversed using the homologous

chromosome as the template. *P*-element excisions are repaired mainly by the second mechanism (Engels et al., 1990; Engels et al., 1994). For *Minos* excisions, both mechanisms seem to be involved in DNA repair. Gap repair by homologous recombination results in either precise excision of the transposon, or partial deletions of the element, while direct ligation generates footprints that contain nucleotides of the terminal repeats from the transposon (Arca et al., 1997).

III. Materials and methods

Strains and dietary conditions

Flies were reared on standard cornmeal/yeast medium at 25 °C. Our study involves the following strains: *y w*; *P{EPgy2}pr-set7^{EY04668}/TM3, Sb, Ser* (Bloomington stock no. 15761), *y w*; *Ki P{Δ2-3}99B* (Bloomington stock no. 4368), *y w*; *Mi{ET1}GRHRII^{MB00838}* (Bloomington stock no. 22910), *w¹¹¹⁸*; *sna^{Sc}/SM6a, P{hsILMiT}2.4* (Bloomington stock no. 24613), *hs-flp;;UAS-frt-mCD2-stop-frt ()*, *w*, *UAS-prset7;; pr-set7²⁰/TM3, Sb* and *y w*; *actin-gal4/CyO; pr-set7²⁰/TM3, Sb* (gifted from Ruth Steward's lab in Rutgers University).

Generation of Crz mutant

P-element mobilization was conducted to generate *Crz null* mutation (Fig. 2-1). *P{EPgy2}pr-set7^{EY04668}* (for short, *EY04668*), was inserted in the 1st intron of *pr-set7* gene, ~500-bp downstream of the *Crz* transcription termination codon. Flies carrying transposase *y w*; *Ki P{Δ2-3}99B* (for short *Δ2-3*) were crossed with the *P*-element allele. Progenies were cultured at 18°C until pupariation. Fifty male progenies with mosaic eyes (*w⁺* in some of the ommatidium) were then individually crossed to balancer *y w;; Ly/TM6C, Sb, Tb* females. Two male offspring from each cross with white eye and *Ki⁺* phenotypes were individually crossed to balancer females again.

Lines with *P*-element excision were identified as pre-pupal lethality of homozygous progenies. Genomic DNA from those homozygous larvae was used for PCR-based screening of the deficiency of the *Crz* locus. Primer set used were: *Crz2* (5'-TGCCCCTCTTCCTCTTCAC) and *Crz5* (5'-

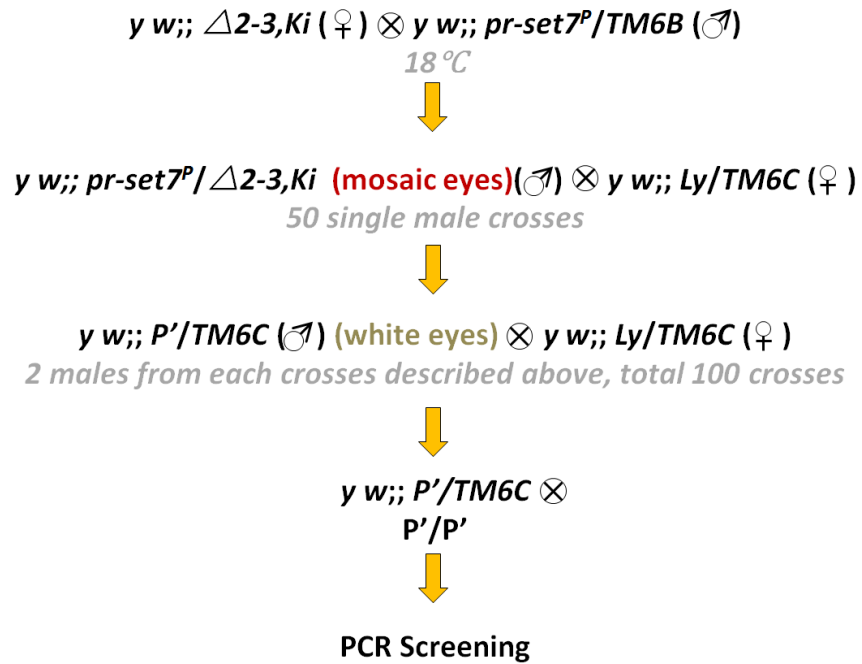


Figure 2-1. Schematic illustration of *P*-element mobilization to induce deficiency in the *Crz* loci.

CTGTCTATCGCAGCTCCAAC) forward primers and *prset7-r2* (5'-GGCGCGTGATCGCTGTGTGG) reverse primer. Sequencing was done at the molecular biology resource facility at the Univ. of Tennessee.

Generation of *CrzR* null mutation

Mobilization of the *y¹ w^{67c23}; Mi{ET1}GRHRII^{MB00838}* was used to induce *CrzR* mutation. Homozygous *GRHRII^{MB00838}* females were crossed to males carrying a genomic source of heat-shock inducible *minos* transposase (*hsLMit^{2.4}*). Progenies were incubated at 37°C water bath for 1 h per day until pupariation. Fifty single male progeny with eyes showing mosaic pattern of green florescence was individually crossed to a balancer *y w;; Ly/TM6C, Sb, Tb* females. Two progeny from each cross with no GFP expression were individually crossed to the balancer again to establish 100 excision lines. Genomic DNA isolated from homozygous flies from each line was used for PCR-based screening of the deletion of the *CrzR* locus. Out cross of the mutant line was performed. First, heterozygous mutant males (*Crz^{Df10A}/TM6C*) were crossed to *wild-type* females. Heterozygous female progenies (*Crz^{Df10A}/+*) were crossed to a balancer *Ly/TM6C* line. Eight male progenies with the balancer were crossed individually to balancer females. Next generation, either *Crz^{Df10A}/TM6C* or *+ /TM6C* flies were self-crossed to generate homozygous progenies.

Genetic rescue of *Crz* null mutation

The genomic DNA region from 500-bp upstream of *Crz* to the 3'UTR end of *pr-set7* gene was cloned by PCR amplification (Fig 2-2). In brief, a 2.9-kb fragment flanking from 500-bp upstream of *Crz* to the 3rd exon of *pr-set7* was amplified using primers *Crz-p500* and *Hind-r*. A 3.25-kb fragment from the 2nd exon to the end of 3'UTR of *pr-set7* gene was amplified using primers *Rescue-f1* and *Rescue-r3* with an engineered *Xba* I site added at the 5'-end of *Rescue-r3* primer. Two genomic fragments were ligated and sub-cloned into *pCasper4* vector (*Crz^{WT}-pr*). Point mutagenesis was introduced into this construct by PCR amplification using reverse complemented primers CZ-

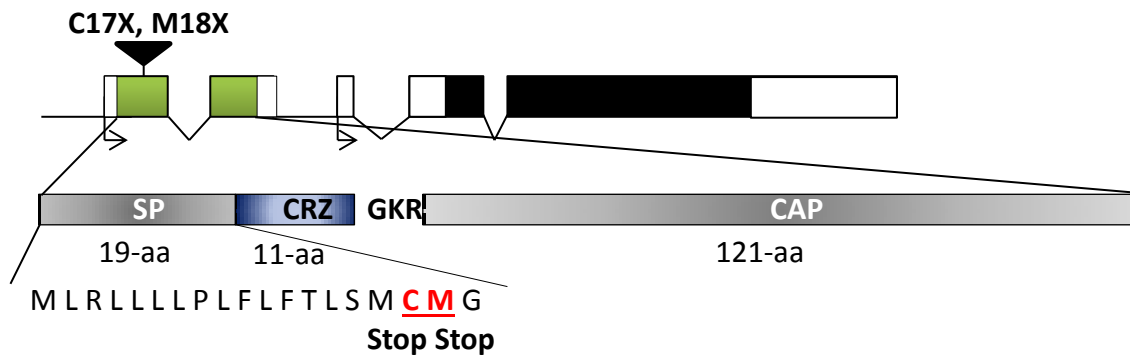


Figure 2-2. Genetic rescue of *pr-set7* in *Crz^{Df10A}* mutants. Constructs carrying full length of *Crz* (Green) and *pr-set7* (Black) genes were PCR amplified. Two termination codons at the end the Crz signal peptide were introduced into the construct via point mutagenesis of C17X and M18X. Abbreviations used: signal peptide, CRZ-associated peptide (CAP), posttranslational processing site (GKR).

muf/CZ-mur (*Crz^{mut-pr}*), where two amino acids were substituted with two termination codons, C17X and M18X. (All primer sequences are provided in Table 2-1).

Immunohistochemistry

CNS from the 3rd instar larvae was dissected and fixed in 4% paraformaldehyde (Sigma F9037). For *Crz^{Df10A}* allele, 2nd instar larvae were used. Specimens were incubated with antiserum against either mature Crz region (obtained from rat, dilution of 1:150), or CAP region (obtained from rabbits, dilution of 1:600). Signals were detected by tetramethyl rhodamine isothiocyanate (TRITC)-conjugated secondary antibody at a dilution of 1:200. Specimens were mounted in a quenching medium (0.5% n-propyl gallate in 90% glycerol and 10 mM phosphate buffer, pH 7.4). Images were acquired with an Olympus BX-61 microscope.

In situ hybridization

CNS from 2nd or 3rd instar larvae was dissected and fixed in PTBw (1xPBS, 0.1% Tween-20) solution with 4% paraformaldehyde and 0.1% deoxycholic acid (DOC). Digoxigenin-labeled *Crz* antisense probe was used at a dilution of 1:50 in hybridization buffer [(50% deionized formamide, 5XSSC, 100 µg/ml tRNA (Sigma R-8759), 100 µg/ml ssDNA, 50 µg/ml Heparin (Sigma H-3393)]. Hybridization was performed in a total volume of 200 µl at 60-62°C for overnight. Anti-dig-alkaline phosphatase (AP) antibody was used at a dilution of 1:2000 in a total volume of 400 µl/well, and AP color reagent [2% AP substrate, 50 mM MgCl₂, 150 mM NaCl, 100 mM Tris (pH 9.5)] was used to detect hybridization signal. The color reaction was stopped by washing 5 times in PTBw.

Fecundity Assay

Adult female fecundity was measured as the number of eggs laid in a 24-hour period for 11 days. Assay was initiated by crossing a single virgin female of *wild-type* or *CrzR⁰¹* to two *wild-type* males. Groups of parent flies were reared on standard food media in vials and kept in a humidified

Table 2-1. PCR primers used for *Crz* and *CrzR* mutant.

	Primer	Sequence 5'-3' (<i>R.E. Site</i>)
Genomic rescue of <i>Crz</i>^{Df10A}	Crz-p500	GTTGTA <u>ACTAGT</u> CCACATATA (<i>Spe</i> I)
	Hind-r	CCTCATCCAT <u>AAGCTT</u> TTGTTCTGC (<i>Hind</i> III)
	Rescue-f1	GATCGGGAATTCCTG
	Rscue-r3	GATCTAGAGGGACAGGTGCGAGGGGGAG (<i>Xba</i> I)
Point mutagenesis	CZ-muf	CGCTCTCCATGTGATAGGGCCAGACCTTC
	CZ-mur	GAAGGTCTGGCCCTATCACATGGAGAGCG
<i>CrzR</i>⁰¹ RT-PCR	59-328 f	TCCCTAAACCACCGTCCAAAC
	59-328 r	ATCCATCACGTGCTCCGGAA

chamber. The parents were transferred to new food vials every day; the numbers of eggs laid were recorded.

Circadian rhythms

A total of twenty-five flies were entrained for 3 days of 12-h light: 12-h dark cycles (LD), and then proceeded into constant darkness conditions (Moxon et al.) for another 7 days. Locomotor activity of individual flies was recorded every 30 min using *Drosophila* activity monitors (Bahn et al., 2009). Data analysis was conducted using ClockLab software (Actimetircs) as described previously (Bahn et al., 2009).

IV. Results

IV-1. Generation of a *null* mutation of *Crz*

To study the function of the neuropeptide *Crz*, characterizations of a complete loss of function allele is absolutely necessary. However, there is no such *Crz* mutant available. In order to obtain a *Crz null* allele, we took advantage of a *P*-element insertion mutation, *P{EPgy2}pr-set7^{EY04668}*. This *P*-element was inserted in the 1st intron of a nearby gene *pr-set7*, approximately 500-bp downstream of *Crz* coding region.

P-element excision was performed according to Robertson *et al.* (Robertson et al., 1988) (Fig. 2-1). Flies heterozygous of *pr-set7^{EY04668}* were crossed with flies carrying the transposase $\Delta 2-3$. Flies heterozygous of excision were self-crossed. As homozygous *pr-set7* mutation is pre-pupal lethal (Karachentsev et al., 2005). Excision lines showing prepupal lethality were considered potentially imprecise excision lines (Fig 2-3 C).

PCR of genomic DNA identified two putative line lacking the *Crz* locus and they are designated: *Crz^{Df10A}* and *Crz^{Df11B}*. From the *Crz^{Df10A}* allele, primers flanking the deletion region produced PCR products that were approximately 950 bp shorter than the *wild-type* sizes (Fig. 2-3B). Sequence

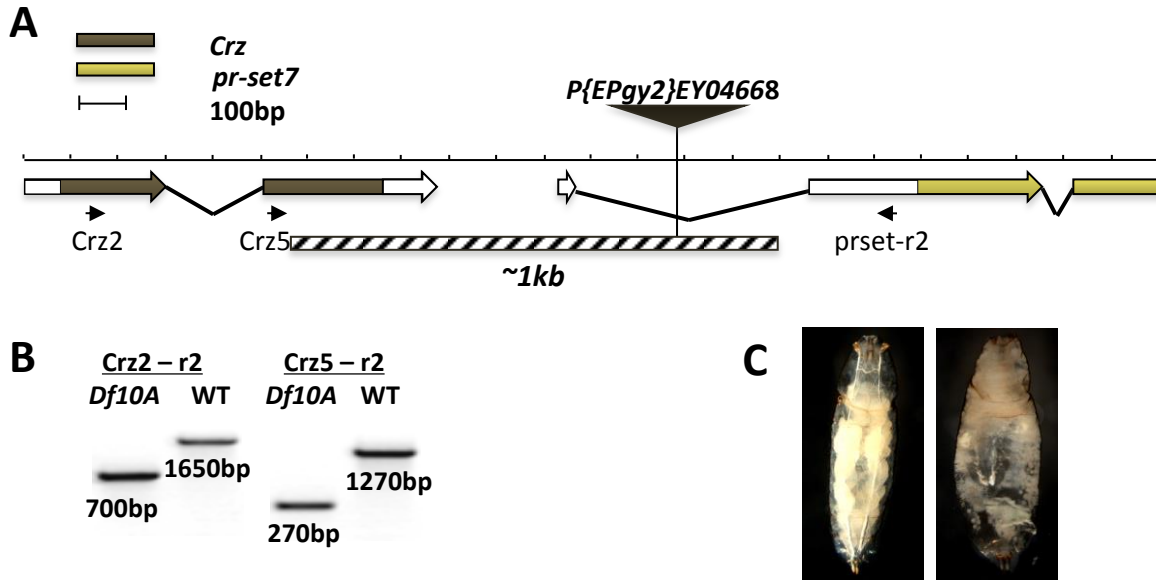


Figure 2-3. P-element mobilization induced deletion in *Crz* loci. (A) *Crz*^{Df10A} deficiency of approximately 950-bp was generated via imprecise *P*-element mobilization, which flank from the 2nd exon of *Crz* gene to the 1st intron of *pr-set7* gene. Exons of *Crz* and *pr-set7* are shown with arrows, with the coding region in black and green for *Crz* and *pr-set7*, respectively. *P*-element insertion is represented by the black triangle. (B) PCR revealed the deletion in *Crz*^{Df10A} allele. Products from the mutants are around 950bp shorter than the ones from the *wild-type*. (C) *Crz*^{Df10A} allele (right) was pre-pupal lethal due to the *pr-set7* mutation.

analysis of *Crz^{Df10A}* confirmed the deletion flanking from the 2nd exon of *Crz* to the 1st intron of *pr-set7* (Fig. 2-3A). For the *Crz^{Df11B}* allele, no PCR product was produced from any of the primer combinations, indicating much larger deletion than *Crz^{Df10A}*.

A series of genetic outcrosses was performed to remove deleterious mutations accumulated during transposable element mobilization. Flies carrying *Crz^{Df10A}* homozygous mutation were lethal at early pupal stage, similarly for the *pr-set7* homozygous mutants, indicating no deleterious mutations accumulated during crossing steps.

Immunohistochemistry confirmed the lack of Crz peptide from the homozygous *Crz^{Df10A}* and *Crz^{Df11B}* 3rd instar larval CNS (Fig. 2-4A-C). *In situ* hybridization also showed the absence of Crz transcripts in the larval CNS (Fig. 2-4D, E). Complementation tests showed that most *Crz^{Df10A}/Df* animals died at the 3rd instar larval stage, which was more severe than the lethality of *Crz^{Df10A}/pr-set7^{EY04668}* flies (Fig. 2-4F, G). Immunohistochemistry using anti-CAP also showed normal Crz expression from the *Crz^{Df10A}/pr-set7^{EY04668}* larvae, but not from the *Crz^{Df10A}/Df* animals, verifying that lack of Crz expression is caused by 10A deficiency.

We then speculated whether Crz peptide expression is required for the development of Crz neurons. To test this, Crz neurons were visualized by reporter gene expression using *Crz-gal4*, and the Crz peptide labeled by anti-CAP. As a result, intact Crz neurons were observed by *Crz-gal4* driven GFP expression, but no Crz immunoreactivity was detected (Fig. 2-5). This result indicates that the lack of Crz neuropeptides in *Crz^{Df10A}* flies has no effect on the formation and development of the Crz neurons.

IV-2. Transgenic rescue of *pr-set7* in *Crz^{Df10A}*

Because Crz –cell ablation flies are developmentally normal, we attempted to rescue *pr-set7* mutation by transgene expression. We took advantage of two *gal4* and UAS lines: *w, UAS-prset7*; *pr-set7²⁰/TM3,Sb* and *yw; actin-gal4/CyO; pr-set7²⁰/TM3, Sb*, where *pr-set7²⁰* is a null allele of *pr-set7*.

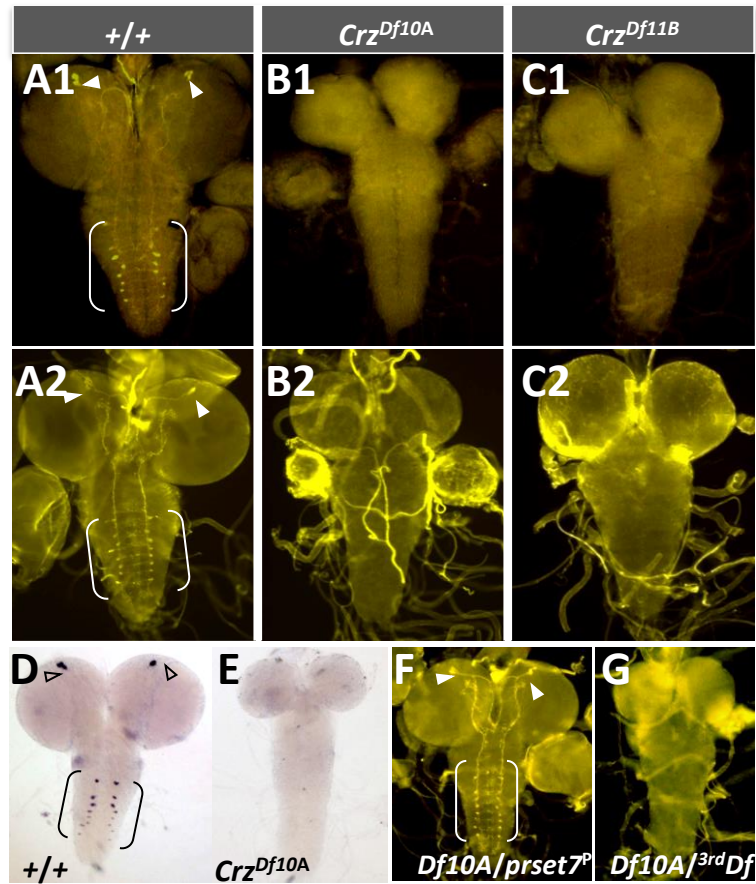


Figure 2-4. Characterization of *Crz^{Df10A}* mutants. (A-C) Immunofluorescence of 3rd instar larval CNS from (A) *wild-type*, (B, C) *Crz^{Df10A}* and *Crz^{Df11B}* with antibodies against (1) mature Crz or (2) the CAP region. No Crz expression was detected from both *Crz^{Df10A}* and *Crz^{Df11B}* mutants. (D, E) *In situ* hybridization of (D) *wild-type* and (E) *Crz^{Df10A}*. No Crz transcript expression was observed from *Crz^{Df10A}* mutants. (F, G) Complementary test of the *Crz^{Df10A}* allele. No Crz expression was detected from (G) *Crz^{Df10A}/3rd(Df)*, while (F) *Crz^{Df10A}/pr-set7^{EY04668}* showed normal Crz expression.

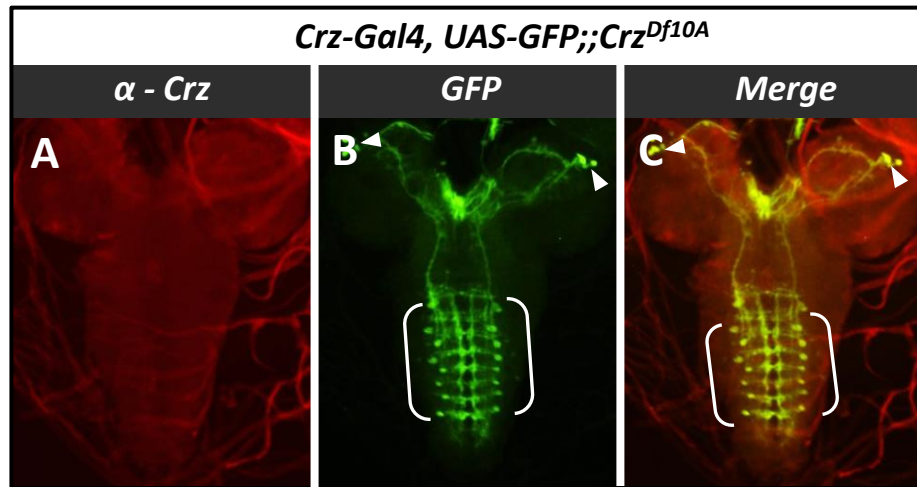


Figure 2-5. Lack of Crz peptide expression does not affect the development of Crz neurons. 3rd instar larval CNS from *Crz^{Df10A}* allele: **(A)** Immunofluorescence using α -Crz, **(B)** GFP expression under the control of *Crz-gal4*, **(C)** merged.

We substituted the *pr-set7²⁰* mutation with *Crz^{Df10A}* to universally express PR-Set7 in *Crz^{Df10A}* background. The rescued flies were viable, but male sterile. Immunohistochemistry was performed on those transgenic rescued *Crz^{Df10A}* mutants. CNS were dissected at the 3rd instar larval stage, 6 hours after pupa formation (APF) and 36 hours APF, with no Crz expression detected from the mutants (Fig. 2-6).

IV-3. Genetic rescue of *pr-set7* in *Crz^{Df10A}*

In order to rescue the lethality caused by *pr-set7* mutation, a construct carrying 500-bp of *Crz* promoter and full length of *Crz* and *pr-set7* gene was generated (*Crz^{WT}-pr*). To introduce a point mutation into the wild-type construct, PCR-based site directed mutagenesis was performed using primers that substitute two amino acid of *Crz* for two premature termination codons. This *Crz* mutant construct (*Crz^{mut}-pr*) carries C17X and M18X mutations within the *Crz* precursor, at the immediate up-stream of the mature *Crz* peptide (Fig. 2-2). The *wild-type* and mutant constructs were introduced into the *Crz^{Df10A}* allele separately. Four transgenic rescue lines with mutant constructs were obtained. *Crz^{mut}-pr(7)*;; *Crz^{Df10A}* (*mut^{X7}*) and *Crz^{mut}-pr(11)*;; *Crz^{Df10A}* (*mut^{X11}*) with the insertion on the X chromosome; *Crz^{mut}-pr(4)*; *Crz^{Df10A}* (*mut^{II4}*) and *Crz^{mut}-pr(20)*, *Crz^{Df10A}* (*mut^{III20}*), with the insertions on the 2nd and 3rd chromosomes, respectively. Two *wild-type* transgenic lines were established: *Crz^{WT}-pr(61)* ;; *Crz^{Df10A}* (*WT^{X61}*) and *Crz^{WT}-pr(44)* ;; *Crz^{Df10A}* (*WT^{X44}*), both have the insertion on the X chromosome.

Immunohistochemistry of the third instar larval CNS confirmed the absence of *Crz* expression from all four mutant alleles (Fig. 2-8B-E); while *Crz* expression was observed from *wild-type* alleles (Fig. 2-7F, G). *Crz* transcript expression was also observed from both *mut^{X7}* and *mut^{X11}* alleles (Fig. 2-7H-J), indicating that the point mutations does not disrupt *Crz* and *pr-set7* transcription.

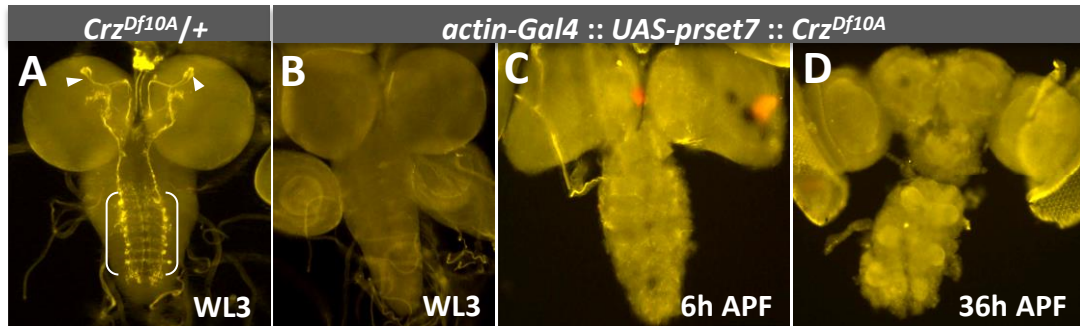


Figure 2-6. Transgenic rescue of *pr-set7* in *Crz^{Df10A}* mutants. (A) Crz expression was detected from 3rd instar larval (WL3) CNS of the heterozygous *Crz^{Df10A}* allele. (B-D) No Crz expression from the transgenic rescued flies at (B) 3rd instar larval stage (WL3), (C) 6 hours APF and (D) 36 hours APF.

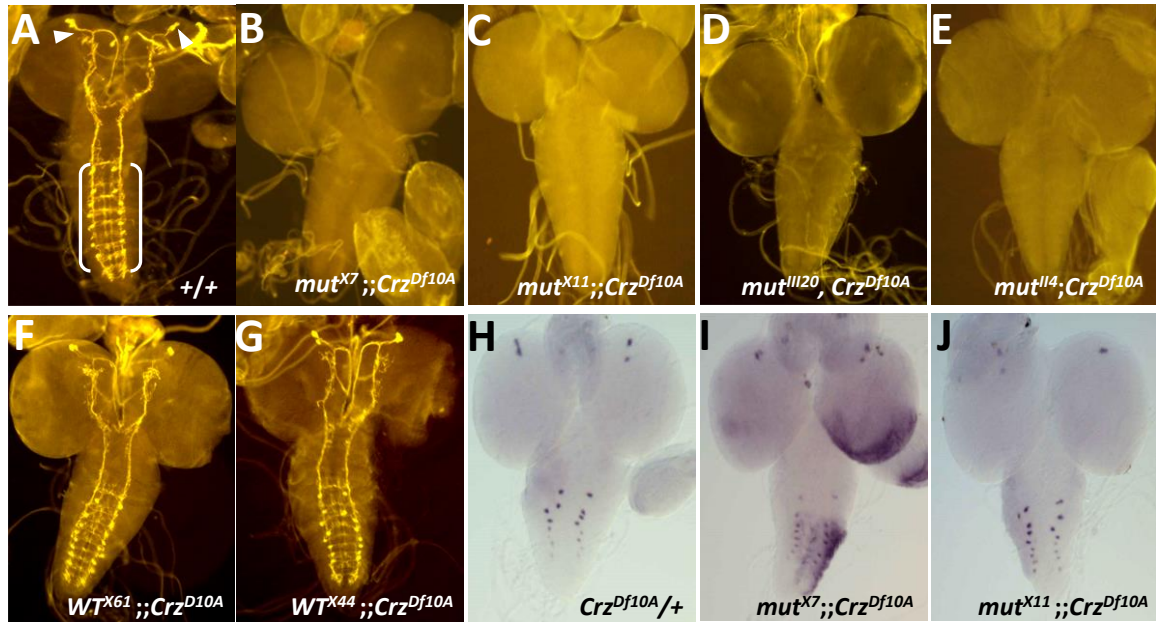


Figure 2-7. Characterization of genetic rescued *Crz^{Df10A}* alleles. **(A-G)** Immunohistochemistry using α -CAP. **(A)** is the wild-type control. **(B-E)** No *Crz* peptide expression was detected in the 3rd instar larval CNS from the *Crz^{Df10A}* allele with mutated *Crz* construct. **(F, G)** *Wild-type* *Crz* expression was observed from the *Crz^{Df10A}* allele with *wild-type* *Crz* construct. **(H-J)** *In situ* hybridization of *Crz* transcript. *Wild-type* *Crz* transcript expression was observed from both **(H)** wild-type control and **(I, J)** *Crz^{Df10A}* allele with *wild-type* *Crz* construct.

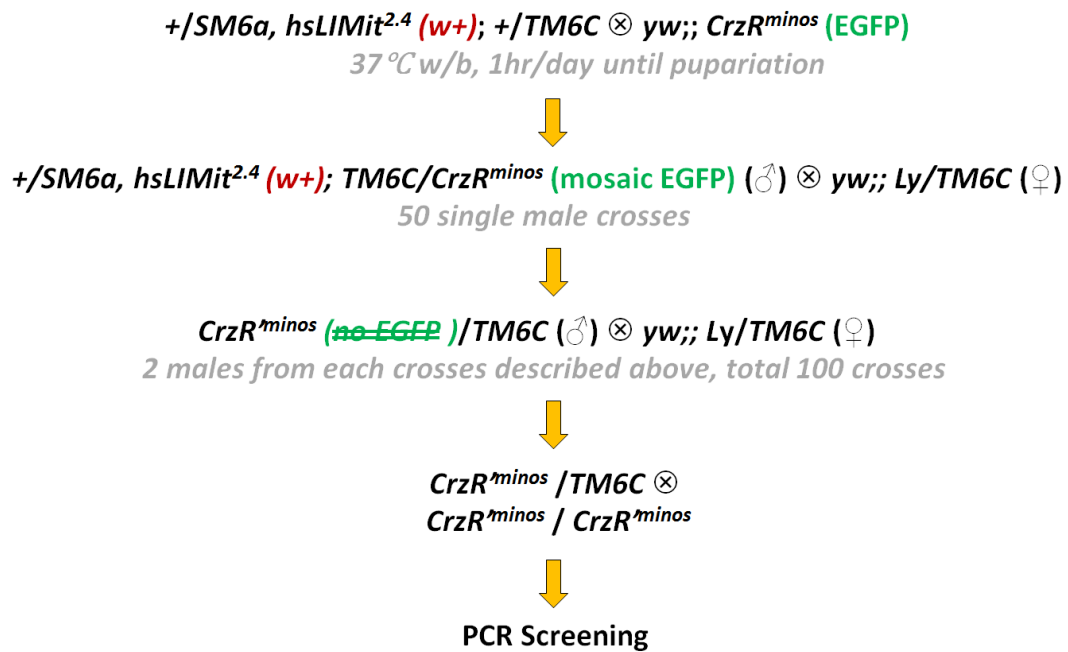


Figure 2-8. Schematic illustration of *Minos*-element mobilization to generate *CrzR null* allele.

IV-4. Generation of a *null* mutation of *CrzR*

In addition to *Crz* mutant, we attempted to obtain a null mutant lacking the *Crz* receptor (*CrzR*, a.k.a. *GRHRII*, *CG10698*). To accomplish this, we first characterized a putative hypomorphic mutant allele *Mi{ET1}GRHRII^{MB00838}* (for short, *MB00838*) that carries a *Minos* transposable element inserted in the fifth intron (Flybase; Fig. 2-9A). The *MiET1* element includes eye-specific GFP marker (Metaxakis et al., 2005).

To generate a *CrzR* null mutant, we mobilized *MB00838* using heat-shock-induced expression of *Minos* transposase, as described in Materials and Methods (Fig. 2-8) (Metaxakis et al., 2005). Mobilization of this element was confirmed by a mosaic pattern of GFP expression in the eyes. Subsequently, we established fifty GFP-negative lines and screened them by PCR to find a mutant deficient of the *CrzR* open reading frame. Preliminary screening identified a candidate and we further refined PCR to define breaking points of this deletion. As shown in Fig. 2-9A, we found a 8-kb deletion including second-to-fifth exons. Interestingly, PCR analysis revealed a footprint of the *Gal4* coding region derived from the *MB00838* element at the original insertion site. RT-PCR targeting 5' region of *CrzR* (-59~+328) confirmed the absence of *CrzR* expression, suggesting that this is a *bona fide* null mutant (Fig. 2-9C). We refer to this mutant as *CrzR⁰¹* as the first amorphic allele of this gene.

Homozygous *CrzR⁰¹* mutants are viable and fertile and have no discernible morphological or developmental aberrations. The mutant females also showed fecundity comparable to wild-type (Fig. 2-10). In addition, mutant flies displayed robust circadian locomotor activity rhythms, and their overall activity levels were similar to those of *wild-type* (Fig. 2-11).

V. Discussion

PR-Set7/Set8/KMT5a is the sole monomethyltransferase (Oda et al., 2009) of H4K20 (H4K20me1). Thus the activity of PR-Set7 is essential for viability. In mice, *pr-set7* knock-out leads to lethality at

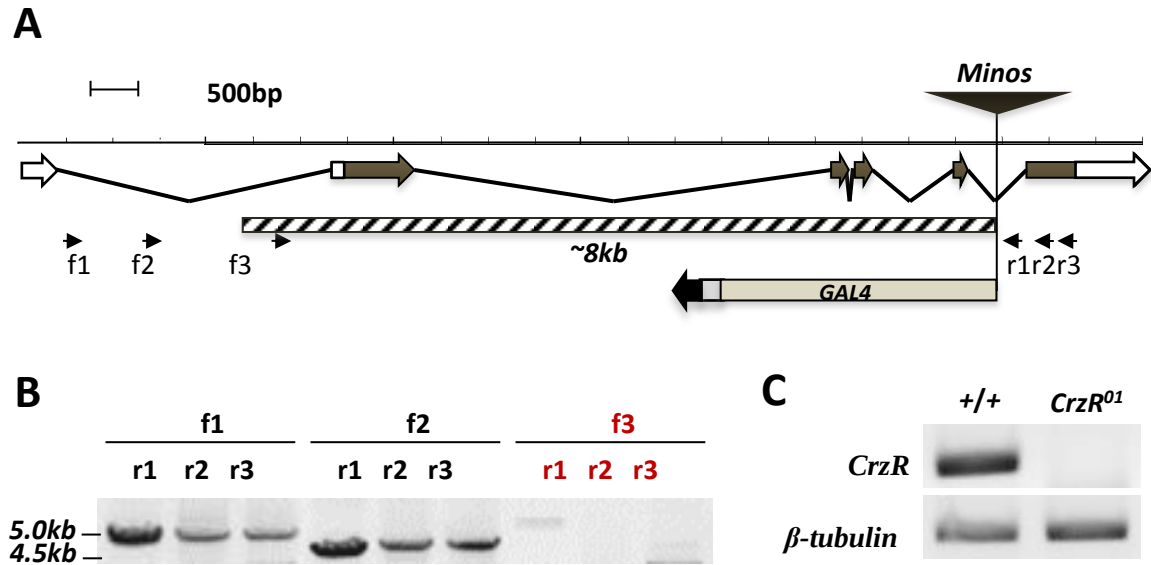


Figure 2-9. Generation of *CrzR*-null allele. (A) Diagram of *CrzR* encoding exons (12427 bp) and approximate locations of primers used for the mutant screening. Exons and introns are shown as arrows and solid lines, respectively. Coding region is shown in grey, and UTRs in white. A deletion of approximately 8-kb including second-to-fifth exons, was indicated by a box with diagonal lines. A footprint of a *Gal4* coding region derived from the *Minos* element was found at the original insertion site. (B) PCR products derived from the designated primer sets. No PCR product was produced from the f3 primer. (C) RT-PCR. No PCR product was detected from *CrzR*⁰¹ allele using 59-328 primer set, while the wild-type produced a product with expected size.

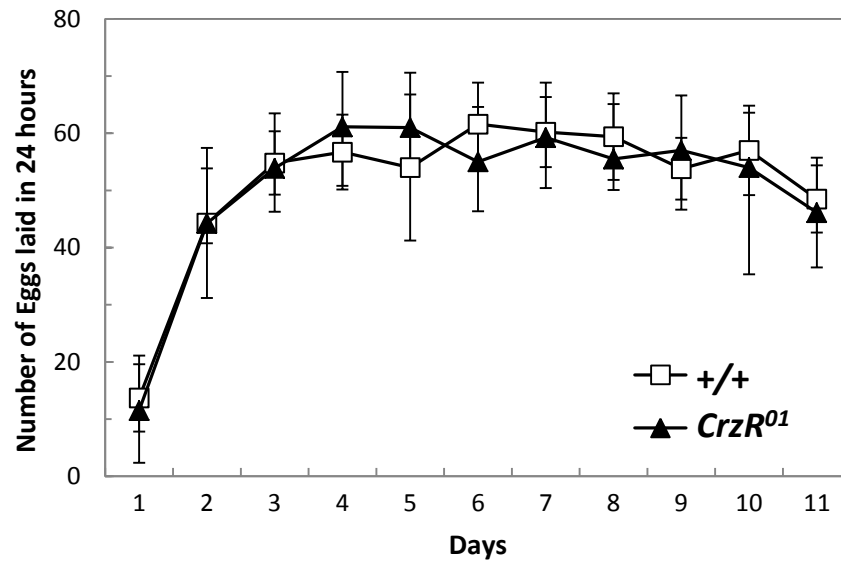


Figure 2-10. Fecundity assay. Numbers of eggs laid per fly were recorded per day (n=8). No difference was found between *CrzR⁰¹* and wild type females.

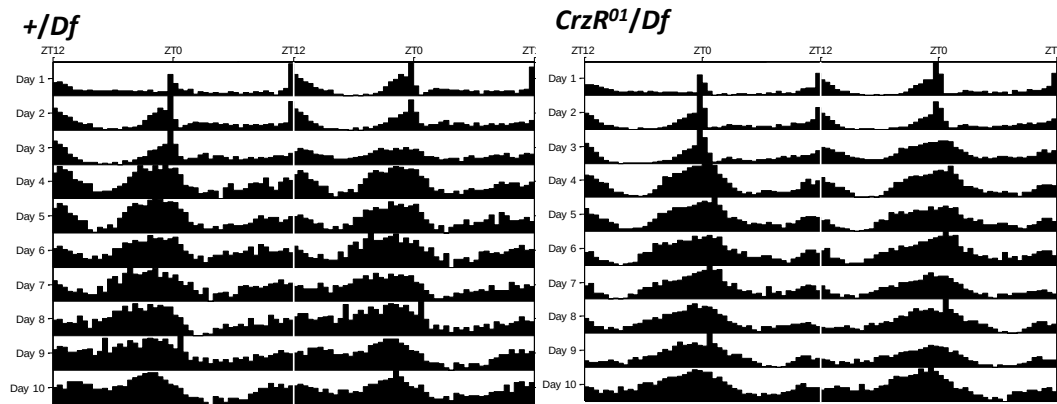


Figure 2-11. Actograms showing circadian locomotor activity rhythms. Flies were entrained for 3 days of LD followed by 7 days of DD. The majority of *CrzR⁰¹/Df* (23/25; 92%) and *Df/+* (26/27; 96%) flies showed normal circadian rhythmic activities, with the period length of 23.88 ± 0.05 for *CrzR⁰¹/Df* and 23.70 ± 0.07 for *Df/+*. Data is represented by average and sem.

embryonic stage and arrest between the four- and eight-cell stage (Oda et al., 2009). Such developmental arrest is possibly due to the accumulation of DNA damage and the disruption of the cell cycle in embryonic stem cells. In *Drosophila*, with maternal contribution of *pr-set7* transcript, loss of *pr-set7* results in lethality at the third instar larval stage, potentially through defects in embryonic patterning (Karachentsev et al., 2005). Another potential role for *pr-set7* is repression of transcription. In *Drosophila*, H4K20me1 was found to localize exclusively to regions on the polytene chromosomes lacking active transcription (Nishioka et al., 2002). Deletion of *pr-set7* also causes a suppression of the variegated phenotype in *Drosophila*, indicating a role of PR-Set7 involving the silencing of gene expression (Karachentsev et al., 2005).

In this study, the lethality due to a double deletion of *pr-set7* in *Crz^{Df10A}* mutant allele has been completely rescued by introducing the genomic sequence of *pr-set7*. However, it is not clear whether the function of *pr-set7* in regulating transcript expression has been completely restored. To solve this problem, a fine analysis of the *pr-set7* promoter region and the distribution of PR-Set7 on the polytene chromosome are required. The rescue construct that we made may not completely restore its function, since none of the *Crz^{mut}-pr* alleles display similar phenotype as compared to the *Crz*-CD flies (data not shown).

In this study, we have also confirmed that lacking of *Crz* neuropeptide does not affect the development of *Crz* neuron, since the intact *Crz* neuron was observed from the *Crz* mutant. This finding is important as if the *Crz* neuron was gone due to the lack neuropeptide, then such loss of function mutants will be no different with the cell ablation mutants. Cell ablation would cause much more severe effects than the single gene mutation, since it would also remove other neuropeptides or neurotransmitters from the system. Similarly, such phenomenon can also be seen from other peptidergic neurons, including *pdf* and *Bursicon* neurons (Lee et al., 2013; Renn et al., 1999). Together these results suggest that the peptidergic neurons develop normally with or without the peptide expression.

The putative hypomorphic mutant allele *Mi(ET1)GRHRII^{MB00838}* may produce truncated CrzR protein., since PCR product was observed when targeting on the 5' end of the gene. No PCR product was produced from the 3' end, indicating the C-terminal truncated CrzR from the *MB00838* mutants. The function of such truncated protein is unclear, since CrzR is a GPCR, and the remained N-terminal region and the transmembrane domains may be capable of agonist binding and signal transduction.

So far most of the studies on Crz signaling focused on Crz neuropeptide, by using either *Crz* cell ablation or *Crz RNAi* techniques. However, little has been done about its receptor. The newly generated *CrzR⁰¹*, which is a null mutant, provide appropriate material for the receptor study. Also, the normal circadian rhythm and female fecundity of this mutant suggests that *Crz* signaling has little possibility to play a role in these biological processes.

Chapter 3

Crz signaling in ethanol metabolism

This study has been submitted for publication.

I. Abstract

Impaired alcohol metabolism can lead to various alcohol-related health problems. Two major enzymes involved in alcohol metabolism are alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH). However, signaling pathways that regulate the activities of these two enzymes remain unknown. In this paper, we have identified *Corazonin* (*Crz*) neuropeptide-expressing neurons and Corazonin receptor (*CrzR*) as important physiological regulators for the recovery from ethanol-induced sedation and ethanol metabolism in *Drosophila*. *Crz*-cell deficient (*Crz*-CD) flies display significantly delayed recovery from ethanol-induced sedation and even more severely impaired recovery was observed in *CrzR*-null mutant flies that we generated. Faster accumulation of acetaldehyde in *CrzR*-null mutants during ethanol sedation indicates defective alcohol metabolism in the mutant tissues. Consistent with this, *Crz*-CD or *CrzR* mutation significantly lowered ALDH activity. In contrast, *CrzR* mutation, but not *Crz*-CD, elevates ADH activity via transcriptional upregulation in a manner dependent on the PKA pathway. Together we propose that *CrzR* is critical in ethanol metabolism through its effects on ALDH ADH activities.

II. Introduction

Alcohol is one of the most commonly abused beverages in the world. Chronic alcohol consumption causes serious health risks such as liver cirrhosis and various types of cancer (Bagnardi et al., 2001). Alcohol metabolism involves a sequence of reactions in which alcohol is first oxidized into acetaldehyde by ADH and then further oxidized into acetate by mitochondrial ALDH. Acetaldehyde promotes adduct formation, leading to the dysfunction of various key proteins and DNA damage (Setshedi et al., 2010). As a result, accumulation of acetaldehyde introduces significant toxic effects during alcohol ingestion, resulting in alcohol-associated health

problems, including cancer. Therefore, the regulatory mechanisms of ethanol metabolism are important to understand the pathophysiological influence of alcohol.

Metabolic rate of alcohol in individuals is greatly influenced from the genetic polymorphism, which gives rise to variants of enzymes with different biochemical properties (Chen et al., 2009; Edenberg, 2007). In addition to genetic factors, transcriptional regulations (Crabb et al., 2001; Dannenberg et al., 2005; Nebert et al., 1990; Yu et al., 1994) and post-translational modifications (Song et al., 2011) affect the activity of ADH/ALDH. Interestingly, hormones, such as thyroid and growth hormone (Hillbom and Pikkarainen, 1970; Israel et al., 1975; Mezey and Potter, 1979; Potter et al., 1989; Ylikahri and Maenpaa, 1968), modulate ADH activity, indicating that endogenous physiological factors could determine the rate of alcohol detoxification.

Genetic bases of alcohol-induced behaviors have been actively investigated in fruit flies, *Drosophila melanogaster*, since progressive behavioral patterns in response to acute alcohol exposure are quite similar to those of humans (Singh and Heberlein, 2000). Moreover, advancements of highly sophisticated genetic and transgenic toolkits available for this species make it an attractive model system to identify and investigate the molecular genetic mechanisms underlying pathophysiological and behavioral outcomes of alcohol consumption in vertebrates.

Various studies have reported that neuropeptides are involved in the regulation of alcohol-related behavior in *Drosophila*. Flies deficient in neuropeptide F (NPF) signaling and ablation or inactivation of corazonin (Crz) neurons showed reduced sensitivity to alcohol-induced sedation (Wen et al., 2005), while the opposite effect was observed with flies deficient of a neuropeptide homologous to mammalian pituitary adenyl cyclase activating peptide (PACAP) and insulin signaling (Corl et al., 2005; McClure and Heberlein, 2013; Moore et al., 1998). *These studies suggest that peptidergic networks play an important role in modulating sensitivity to alcohol.*

We have long been interested in biological roles of the neuropeptide corazonin (Crz) that was originally found in the American cockroach (Veenstra, 1989). Although the sequence and structure of Crz is highly conserved among different insect species (Sha et al., 2012), it has been shown to

affect diverse physiological functions in a species-specific manner: cardio-acceleration in the cockroach (Veenstra, 1989), induction of cuticular pigmentation in the migratory locust (Tawfik et al., 1999), reduction of the spinning rate and pupal development in the silkworm (Tanaka et al., 2002), and induction of ecdysis in the moth (Kim et al., 2004a). In *Drosophila*, Crz is produced by a group of neuroendocrine cells in the pars lateralis of the protocerebrum (Choi et al., 2005; Lee et al., 2008). Given the complexity of the Crz neuronal architecture, Crz is predicted to deliver multiple biological functions, and Crz neurons are shown to be associated with sperm transfer and copulation duration (Tayler et al., 2012), regulation of trehalose levels (Lee et al., 2008), and responses to physiological stresses (Zhao et al., 2010).

Crz receptor (CrzR) is a member of the Class-A G protein-coupled receptor (GPCR) family (Sha et al., 2012). Using Crz cell ablation and *CrzR*-null mutant, we investigated biological functions of the Crz signaling system in *D. melanogaster*. Here we show that both types of mutant flies displayed significantly delayed recovery from ethanol-induced sedation. We further show that such a 'hangover' phenotype likely results from faster acetaldehyde accumulation due to higher ADH as a result of transcriptional up-regulation and lower ALDH activity in the *CrzR* mutant. However, only the latter event was observed in Crz-cell ablation, indicating complicated signaling mechanisms associated with CrzR.

III. Materials and methods

Fly strains

Flies were reared on standard cornmeal/yeast medium at 25 °C. Alcohol related behavioral and metabolic assays were performed on 3-5-day-old males. Our study involves the following strains: *y w;; CrzR⁰¹* (fly strains and crosses described in chapter 2), *w¹¹¹⁸; UAS-PKAⁱⁿ* (gifted from Ben White at NIH), *w¹¹¹⁸; UAS-PKCⁱⁿ* (Bloomington stock no. 4589), *y w;; UAS-cbz/TM3, Sb, Ser* (Bloomington

stock no. 7222). *w¹¹¹⁸*; *Df(3L)BSC380/TM6C, Sb¹* (for short *Df*; Bloomington stock no. 24404) was used to uncover the *CrzR* locus.

For *CrzR-gal4*, a fragment of 3.5-kb sequence upstream of the *CrzR* (-3130 to +331, +1 indicates transcription start site) was PCR amplified (primer sequences are shown in Table 3-1). This fragment was sub-cloned into the pPTGAL vector at *Xba* I / *Not* I sites for germline transformation. Three independent *CrzR-gal4* lines (Se, T11a and T9) were established, showing consistent patterns of reporter gene expression. The following transgenic lines were used to induce targeted ablation of *Crz* neurons: *y w*; *Crz-gal4* (Lee et al., 2008), *y w*; *UAS-rpr*, *y w*; *UAS-Δrpr*, *y w*; *UAS-grim*, and *y w*; *UAS-hid/CyO, y⁺* (Lee et al., 2013).

For *UAS-CrzR-GFP*, a 1.7-kp of *CrzR* cDNA from +1 ~ +1737(+ 1 indicates translation start site) was PCR amplified using *CrzR-f3/r3* (sequences provided in table 3-1), with engineered restriction enzyme sites *Xho* I and *Hind* III at the 5' -ends. The PCR product was sub-cloned into *pEGFP-N1* vector between at immediate upstream of the *GFP* coding sequence. *pCrzR-GFP* were then transformed into a *dam⁻* competent cell JM110 to demethylate the *Xba* I site , and then sub-cloned into *pUAST* vector via by *Xho* I and *Xba* I sites.

Behavioral assay

Recovery from sedation: Groups of twenty-five males aged 1- to 3-days-old were collected and maintained in a food vial for 1-2 days. Flies were then transferred into empty plastic vials (O.D. x H: 25 x 95mm), which were then sealed with cotton plugs. For ethanol sedation, 1 ml of 100% ethanol was applied onto the cotton plugs. Following 17-18 min of exposure, the cottons were replaced with fresh buzz plugs and then the vials were placed upside down. Flies that were capable of climbing up were considered “recovered”, and the numbers of recovered flies were recorded every 10 min. For ethyl ether sedation, 50 μl of 100% ethyl ether was applied onto the cotton plug. Following 10 min of exposure, the cottons were replaced with fresh buzz plugs and then the vials were placed upside down. Flies that were capable of climbing up the vial were

Table 3-1. PCR primers used.

	Primer	Sequences (5' – 3') (<i>R.E. Site</i>)
Q-RT-PCR	Adh-f1	GCAGTTCAGCAGACGGGCTAACGAG
	Adh-r1	CACATCATAGGGGTAGAAGGTGAC
	Adh-f2	GTCACCTTCTACCCCTATGATGTG
	Adh-r2	CCAGGAGTTGAACGTGTGCACCAG
	Aldh-f1	GCAGCTGTTGCGAACTACTCCTC
	Aldh-r1	CCATGGGAATAGTCTTGCCATGG
	Aldh-f2	CCACAGACAACGCCGATATCCTC
	Aldh-r2	GCGGGTGTAAGTGAAGAAATCTCCG
	β -tubulin -f	GCAACAACTGGCCAAGGGTCATTAC
	β -tubulin -r	CTTGGCATCGAACATCTGCTGGGTCAG
CrzR Promoter	CrzR-f1	TGTGCAATTCGCAAGAAAA
	CrzR-r1	CTTGCTGCTGCTTTGGTTT
UAS-CrzR-GFP	CrzR-f2	ATATCTCGAGATGGAGGACGAGTGGGGCTCCTTTG (<i>Xho</i> I)
	CrzR-r2	GATCAAGCTT CACGCTCGACACTCGCTCGTTG (<i>Hind</i> III)

considered “recovered”, and the numbers of recovered flies were recorded every 10 min for up to 2 h.

Toxicity assay: This assay was performed according to Barbancho *et al* (Barbancho et al., 1987), with slight changes. Groups of twenty males aged 1- to 3-day-old were collected and transferred into hermetically sealed plastic vials (O.D. x H: 25 x 95mm) containing 3% sucrose medium supplemented with acetaldehyde (0.5%-1%). To specifically inhibit ADH activity in the acetaldehyde-mediated toxicity assay, flies were pre-fed with sucrose medium containing 0.5% acetone for 24 h prior to exposure. The toxicity of acetaldehyde was represented by the number of knocked-down flies as a function of exposure time.

Fecundity Assay: A single wild-type or *CrzR⁰¹* virgin female was crossed to 2 wild-type males, and kept in a food vial for 24 h before assay started. The flies were reared in fresh food vials with dry yeast powder to allow them to lay eggs for 24 h before transferred to new food vials. The number of eggs laid in each vial was recorded every 24 h for up to 10 days. All food vials were kept in a humidified chamber at around 25 °C.

Circadian rhythms: A total of twenty-five flies were entrained for 3 days of 12-h light: 12-h dark cycles (LD), and then preceded into constant darkness (DD) conditions for 7 days. Locomotor activity of individual flies² was monitored using *Drosophila* activity monitors (Bahn et al., 2009). Data analysis was conducted using ClockLab software (Actimetircs).

Subcellular fractionation and enzyme activity assay

A hundred flies from each group were homogenized in 1 ml of isolation buffer [50 mM sodium phosphate (pH 7.4), 0.24 M sucrose, 0.5 mM EDTA, 0.5 mM dithiothreitol, 0.001% (w/v) phenylthiourea] as described (Heinstra et al., 1989). The homogenate was centrifuged for 10 min at 1000 rpm and the supernatant was saved. The pellet was resuspended in 0.5 ml of the isolation buffer. Following centrifugation as before, the supernatant was combined with the first one. The pooled supernatant was centrifuged for 45 min at 14,000 rpm to separate cytosolic and

mitochondrial fractions (Leal and Barbancho, 1993). The pellet was washed with isolation buffer twice and resuspended in 250 μ l of the same buffer supplemented with 1% (v/v) Triton-X-100. The resuspension was allowed to stand for 15 min and then centrifuged at 14,000 rpm for 20 min. The supernatant was used for mitochondrial fraction. All operations were performed on ice except centrifugation at 4°C.

ALDH and ADH activities were assayed spectrophotometrically by measuring reduced β -NAD⁺ at 340 nm. ALDH activity was assayed by the method of Moxon *et al.* (Moxon et al., 1985). Briefly, the assay was started by adding 25 μ l fly extract into 500 μ l of 50 mM Tris/HCl (pH 8.6) including 3.6 mM acetaldehyde, 1 mM β -NAD⁺, and 20 mM pyrazole. The absorbance was recorded every min for up to 20 min by using Smartspect™ 3000 kinetics program (BioRad). ADH activity was assayed after Barbancho *et al.* (Barbancho et al., 1987) by adding 20 μ l homogenate into 500 μ l of 50 mM Tris/HCl (pH 8.6) buffer containing 100 mM 2-propanol, 1 mM β -NAD⁺ and 1.6 mM cyanamide. The absorbance was recorded every 10 sec for up to 3 min.

Succinate dehydrogenase activity was assayed by following the reduction of INT [2-(p-indophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium] (Pennington, 1961). The reaction was initiated by adding 2 μ l of mitochondrial and 20 μ l of cytosolic fractions into 200 μ l substrate solution (0.11% w/v INT, 55 mM sodium succinate, 25 mM sucrose in 100 mM sodium phosphate, PH 7.4), followed by 37°C water bath incubation for 15 min. The reaction was stopped by adding 200 μ l of 3% ice-cold HCl. The chromogen was extracted with 600 μ l ethyl acetate by centrifugation at 14,000 rpm for 5 min, and then its absorption was determined at 490 nm.

Real-time quantitative RT-PCR

Total RNA was purified from 20 adult males using TRIzol reagent (Invitrogen) according to the manufacture's protocols. About 5-10 μ g of total RNA was added to 40 μ l of Go Superscript reverse transcription mix (Promega) to generate cDNA. cDNA was then purified and eluted with 40 μ l water using a PCR purification kit (Qiagen). SYBR Green reaction kit (Bio-Rad) was used for *q*-PCR,

with 0.5 μ l cDNA included in 25 μ l reaction volume. Real-time PCR was carried out using an iQTM5 Multicolor Real-Time PCR Detection System (Bio-Rad). Amplifications were initiated with a 10-min denaturation at 95°C, followed by 40 cycles of 95°C, 15sec $\rightarrow$ 55°C, 30 sec $\rightarrow$ 72°C, 30 sec. Reactions were run in triplicate. Primers (Table 3-1) were designed to flank at least one intron to ascertain that the PCR products were derived from the cDNA. *β -tubulin* was used as an internal control to normalize expression levels of the target genes in different genotypes. Expression data were obtained using the $2^{-\Delta\Delta CT}$ method described by Livak and Schmittgen (Livak and Schmittgen, 2001) where $\Delta\Delta CT$ equals the normalized cycle threshold (ΔCT) of *Aldh* or *Adh* in test flies minus the ΔCT of the same gene in wild-type flies.

Measurement of aldehyde and ethanol content

Forty male flies were homogenized in 1 ml distilled water with 20 mM pyrazole and 1.6mM cyanamide to inhibit ADH and ALDH activities. The homogenates were centrifuged at 14,000 rpm for 20 min at 4°C and the supernatant was kept in ice. Standard solutions were prepared from pure ethanol and acetaldehyde in the same assay buffer.

Acetaldehyde and ethanol contents in the fly extracts were measured using an Agilent 7890 gas chromatograph equipped with Agilent model G1888 headspace auto sampler, a flame ionization detector and a DB-624 capillary column (60 m x 0.32 mm x 1.8 μ m). Separation of acetaldehyde, ethanol was complete under the following conditions; injection volume 0.2 μ l; split ratio 50:1; injection port temperature 200°C; oven temperature program: 60°C for 2 min, 25°C min⁻¹ to 200°C for 1 min, FID temperature 300°C; carrier gas N₂; and gas flow 3.0 ml/min (helium). For the auto sampler: auto sampler oven temperature 70°C; transfer line temperature 125°C; loop temperature 125°C; vial equilibration time 15 min; high shaking (mixing) speed; loop fill time 0.03 min; inject time 0.50 min; vial pressure 10 psig; pressurization time 0.5 min. All determinations were carried out in triplicate.

Microscopy

CrzR-gal4 line was crossed to *UAS-mCD8GFP;; UAS-Redstinger*. The resulting transgenic adults were dissected and incubated in fixative (4% paraformaldehyde in PBS) for 30 min at room temperature. Tissues were washed in PBS (3 x 5 min) and mounted in a quenching medium (0.5% n-propyl galate in 90% glycerol and 10 mM phosphate buffer, pH 7.4). Differential fluorescent signals were captured by Olympus BX-61 microscope equipped with a CCD camera and then merged.

Statistics

Statistical analyses were performed using InStat 2.0 (GraphPad Software). Student's unpaired *t*-test was used to determine significant differences between two means. Difference among multiple means was tested by one-way ANOVA followed by Student-Newman-Keuls (SNK) multiple comparisons test.

IV. Results

IV-1. Hangover phenotype mediated by the Corazonin neurons

Targeted cell ablation was accomplished by inducing transgenic expression of one of the cell death genes (*hid*, *grim* or *reaper*) (e.g., Lee et al., 2008). Flies lacking Crz neurons (Crz-cell deficiency; for short, Crz-CD) showed significantly delayed recovery from ethanol sedation. Flies were completely sedated with vapors from 100% ethanol after 17~18 min, and then were allowed to recover in ethanol-free environment for up to 4 h.

Crz-CD flies generated by ectopic expression of *reaper* (*rpr*) showed significantly delayed recovery with about 40% lethality, while expression of mutant *rpr* (Δrpr , mutated form of *rpr* lacking IBM death domain; Lee et al., 2013) showed a near complete recovery after about 2.5 h (Fig. 3-1). This result is consistent with Crz-CD flies derived from crossing between *Crz-gal4* and *UAS-hid*, which

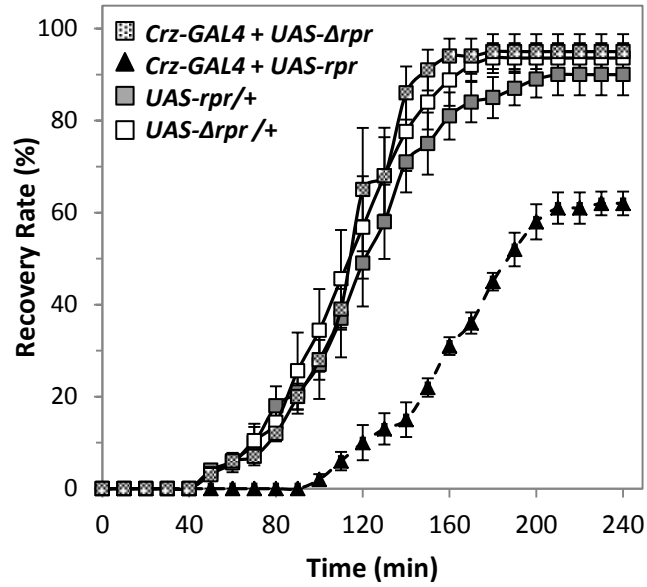


Figure 3-1. *Crz* cell ablation causes hangover phenotype from ethanol-induced sedation. *Crz* neuron ablation by *rpr* transgene expression (triangles) lead to delayed and incomplete recovery compared to wild-type controls (squares). Each data point is a mean ($\pm$ sem) of 3-5 replicates.

showed significantly delayed recovery (Choi, 2009). These results together suggest that *Crz* neurons are required for the recovery from ethanol-induced sedation.

IV-2. Hangover phenotype of the *CrzR⁰¹* mutant

Wild-type and *CrzR⁰¹* mutant flies, both in *y w* background, were subjected to 100% ethanol. When exposed to ethanol for 17.5 min, recovery of *CrzR⁰¹* flies was severely retarded; only 20% was fully recovered after 4 h, whereas around 70% of control flies did (Fig. 3-2A). Similar delayed of recovery was observed from the mutant flies with 18-min exposure (Fig. 3-2B).

To confirm whether the hangover phenotype is caused by the deletion of the *CrzR* locus, we performed similar assays using hemizygous *CrzR⁰¹* (*CrzR⁰¹/Df*) or control (+/*Df*) with *w¹¹¹⁸* background. With 17.5-min exposure, *Df/+* flies were fully recovered after 2 h, while *CrzR⁰¹/Df* flies took 4 h to completely recover (Fig. 3-2C). The recovery of *Df/+* flies was largely unaffected when exposure duration was increased to 18 min, while only 70% of the mutant hemizygous flies recovered by 4 h (Fig. 3-2D). These results overall are consistent with the hangover phenotype that we observed with *Crz*-CD flies, suggesting that *Crz* signaling is causally associated with the hangover phenotype.

We further attempted to rescue *CrzR⁰¹* mutation by transgenic expression of *CrzR* cDNA under *CrzR* driver. *CrzR* cDNA from -59 to 579-bp (+1 indicates the translation start site) was PCR amplified and fused with a GFP tag to its C-terminal, and sub-cloned into *pUAST* vector. The stability of tagged *CrzR* was confirmed by visualizing the GFP signal from a peptidergic neuron driver, *c929*, as three independent *UAS-CrzR-GFP* alleles showed clear GFP expression (Fig. 3-3A). Transgenic expression of *CrzR* in the mutant significantly increased the recovery rate compared to the non-controls (Fig. 3-3B). Six hours after ethanol exposure, 83% of the rescued flies were fully recovered, while the percentage observed from the mutant flies were only 32% and 50%.

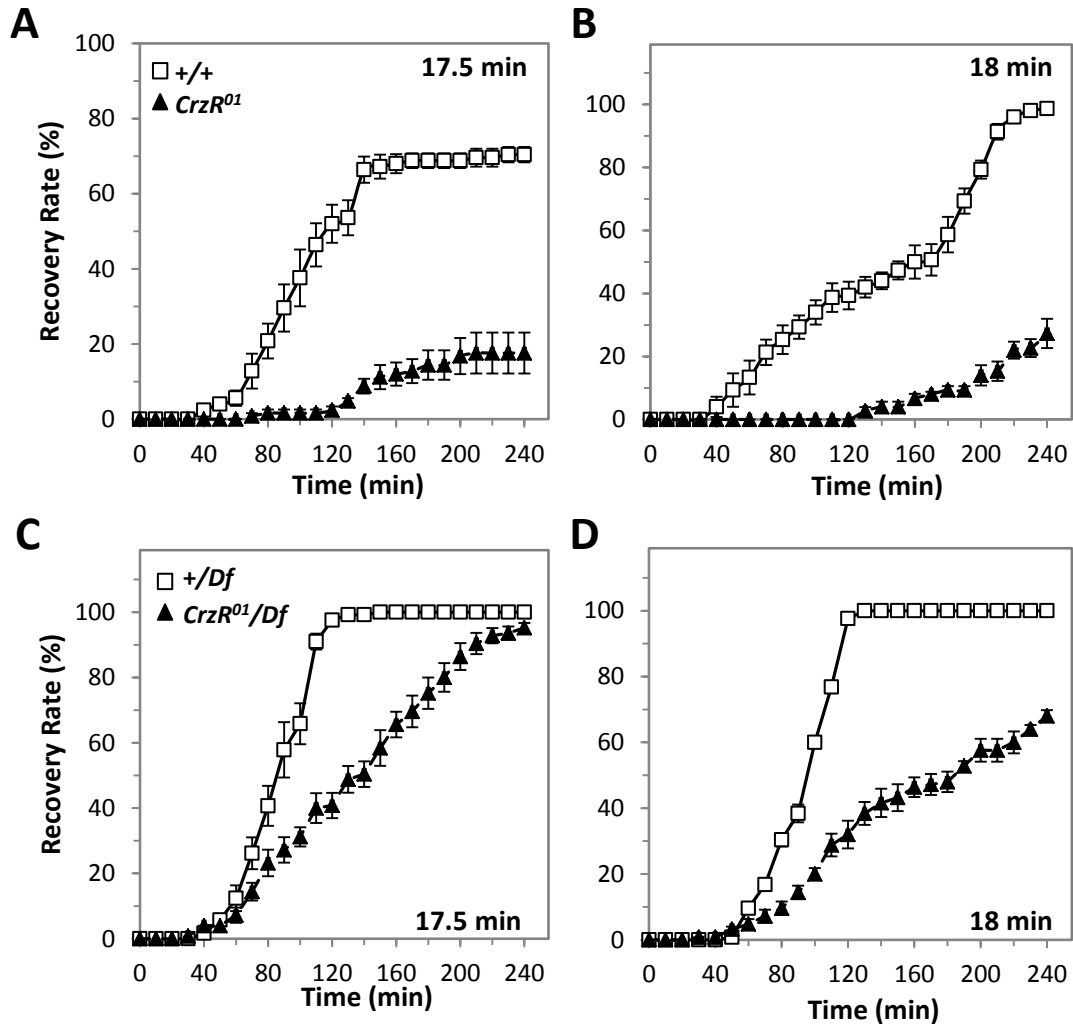


Figure 3-2. Hangover phenotype of the *CrzR⁰¹* mutant flies. Flies were exposed to 100% ethanol for 17.5 min or 18 min as indicated. (A, B) Homozygous *CrzR⁰¹*. (C, D) *CrzR⁰¹/Df*. Each data point is represented by an average and sem of five replicates.

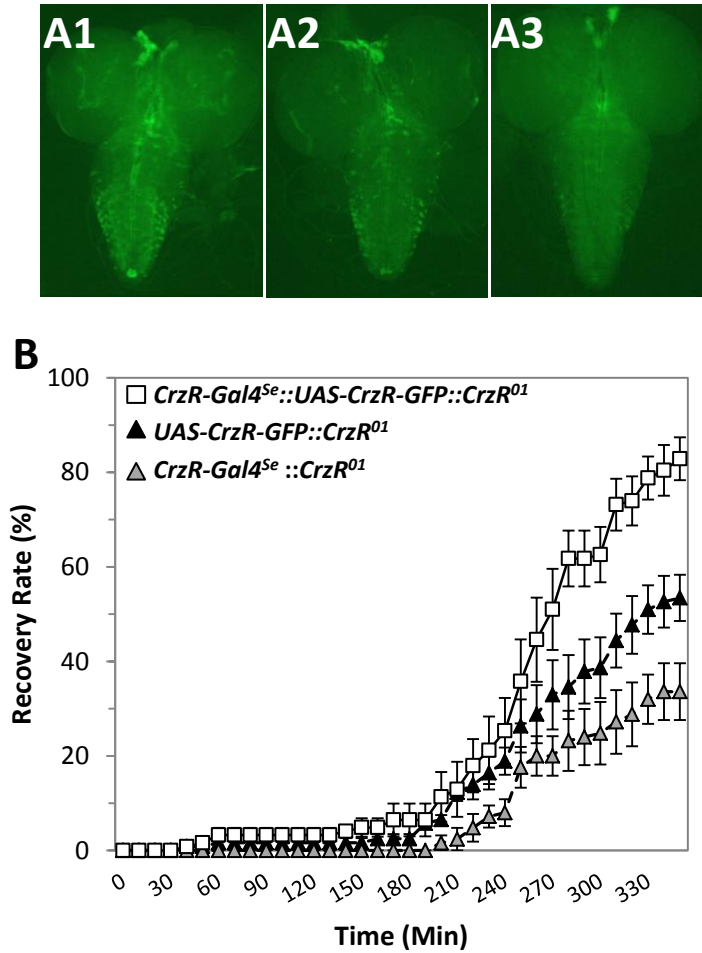


Figure 3-3. Transgenic rescue of *CrzR⁰¹* mutation. (A) Three independent *UAS-CrzR-GFP* alleles were established, each carrying a single copy of transgene on (1) X, (2) 2nd and (3) 3rd chromosome. Expression of *CrzR-GFP* was observed using a peptidergic neuron specific driver (*C929-Gal4*). **(B)** *CrzR⁰¹* carrying hemizygous *UAS* or *Gal4* (triangle) showed significantly delayed recovery from ethanol sedation, with only less than 40% recovered at the end of 6 hours; while 80% of the transgenic rescued *CrzR⁰¹* (square) flies were recovered ($n \geq 3$). Each data point represents mean $\pm$ sem. All genotypes are in *y w* background.

Such hangover phenotype is not a general response to other sedative agents, Since neither *Crz*-ablation nor *CrzR⁰¹* mutation affected the recovery rate as compared to control flies, this supports that the hangover phenotype is likely to be specific to ethanol-induced sedation (Fig. 3-4).

IV-3. Defective acetaldehyde metabolism in *CrzR⁰¹* mutant.

Ethanol first metabolizes into acetaldehyde, which is 30 times more toxic than ethanol, as it forms adducts readily with DNA and various proteins (Setshedi et al., 2010). Hangover symptoms from ethanol consumption are mainly a result from the accumulation of acetaldehyde in humans (Yokoyama et al., 2005). Thus we wondered if defective recovery of *CrzR⁰¹* flies from ethanol-induced sedation is a consequence of abnormal ethanol metabolism. To test this hypothesis, we used gas chromatography to monitor the concentrations of acetaldehyde and ethanol following exposure to 100% ethanol. Remarkably, acetaldehyde and ethanol contents in both *CrzR⁰¹* homozygotes and *CrzR⁰¹/Df* flies were about 2-fold higher than those of control flies 45 min after exposure (Fig. 3-5A).

Faster accumulation of acetaldehyde in *CrzR* mutants is possibly due to the impaired acetaldehyde oxidation as a result of lower ALDH activity. Remarkably, we found that ALDH activity of *CrzR⁰¹* whole fly extract exhibited about 63% of wild-type readings (Fig. 3-6A). Since acetaldehyde enters the mitochondria where it is oxidized into acetate by ALDH, the activity of ALDH was also measured specifically in the mitochondria. Succinate dehydrogenase activity, a mitochondrial marker, was observed almost exclusively in the mitochondrial fraction in both genotypes (Fig. 3-6B), verifying successful separation of the mitochondrial fraction from the cytosol. Around 30% reduction of ALDH activity was observed in the mutant mitochondrial fraction (Fig. 3-6C). Similarly, *Crz*-CD flies (*Crz::hid*) showed significant reduction of whole cell ALDH activity (~55% of wild-type) (Fig. 3-7A) (Choi, 2009) while *grim*-induced cell ablation produced around 30% reduction of ALDH activity in the mitochondrial fraction (Fig. 3-7B). These results together suggest

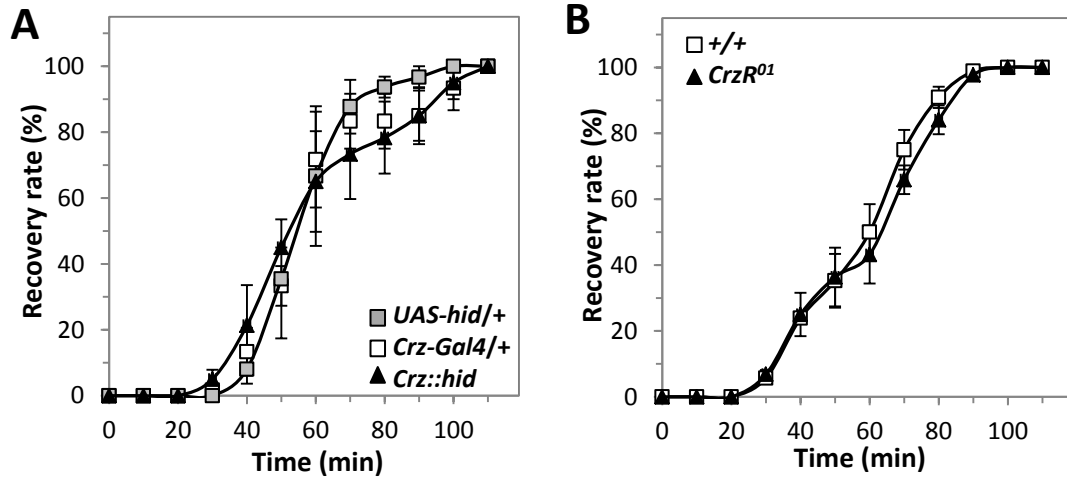


Figure 3-4. Recovery test from ethyl ether-induced sedation. (A) No obvious difference of recovery rate was observed between *Crz*-cell ablated flies (triangle) and controls (square) (n=3). (B) No delayed recovery was observed for *CrzR⁰¹* mutant (n = 4). Each data point represents mean $\pm$ sem. All genotypes are in *y w* background.

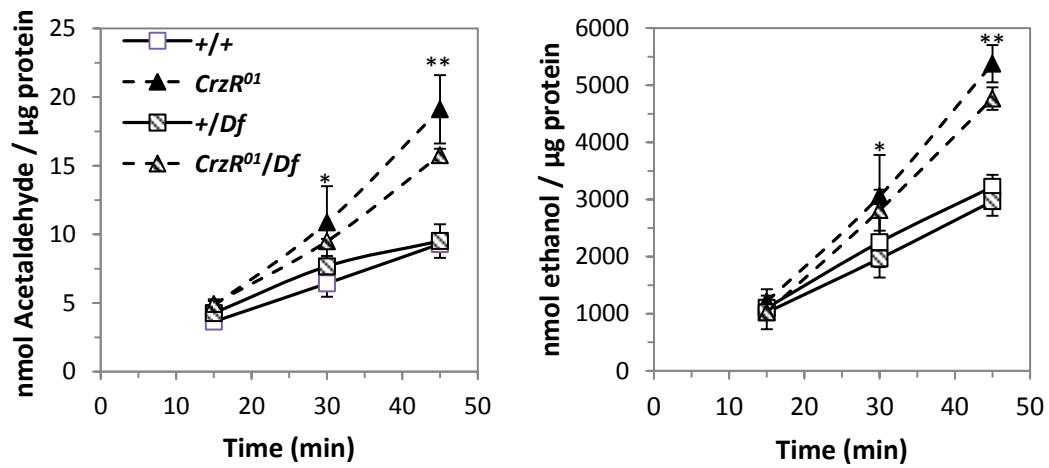


Figure 3-5. Rapid accumulation of ethanol and acetaldehyde. Internal concentrations of ethanol and acetaldehyde from adult males exposed to 100% ethanol. Endogenous levels of ethanol and acetaldehyde is ignorable, because non-treated flies showed less than 0.5% of the levels that were detected from 45-min ethanol treated flies. (For each time and treatment, n=4 represents mean $\pm$ sem. * $p < 0.05$, ** $p < 0.01$).

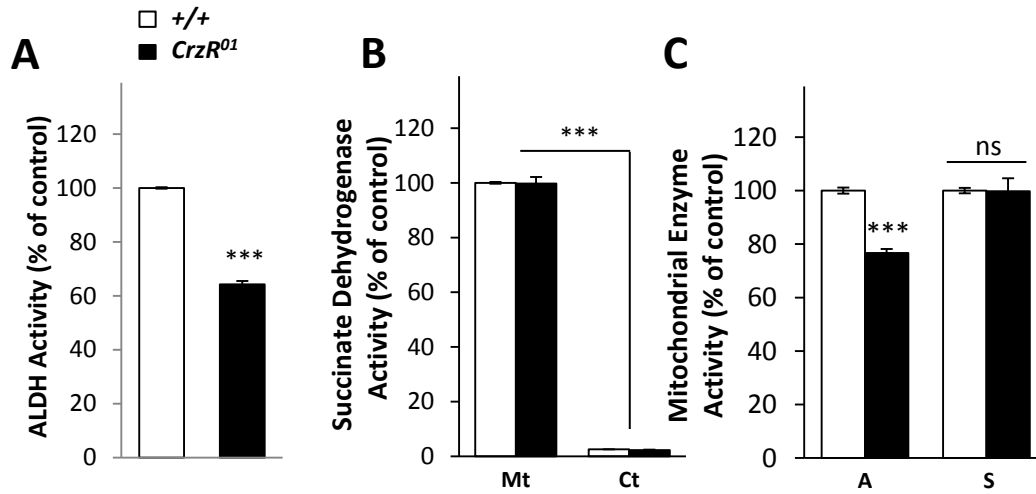


Figure 3-6. Lack of *CrzR* leads to reduced ALDH activity. (A) Deficiency of *CrzR* resulted in the reduction of whole cell ALDH activity (n=3). (B) Succinate dehydrogenase activity in mitochondria (Mt) and cytosol (Ct). The activity was detected almost exclusively in the mitochondrial fraction. (C) Reduction of mitochondrial ALDH activity (A) in *CrzR⁰¹* mutant. Succinate dehydrogenase activity (S) is the same between control and mutant (ns, not significant). Each data point represents mean $\pm$ sem. (n=3. *** $p < 0.001$). All genotypes are in *y w* background.

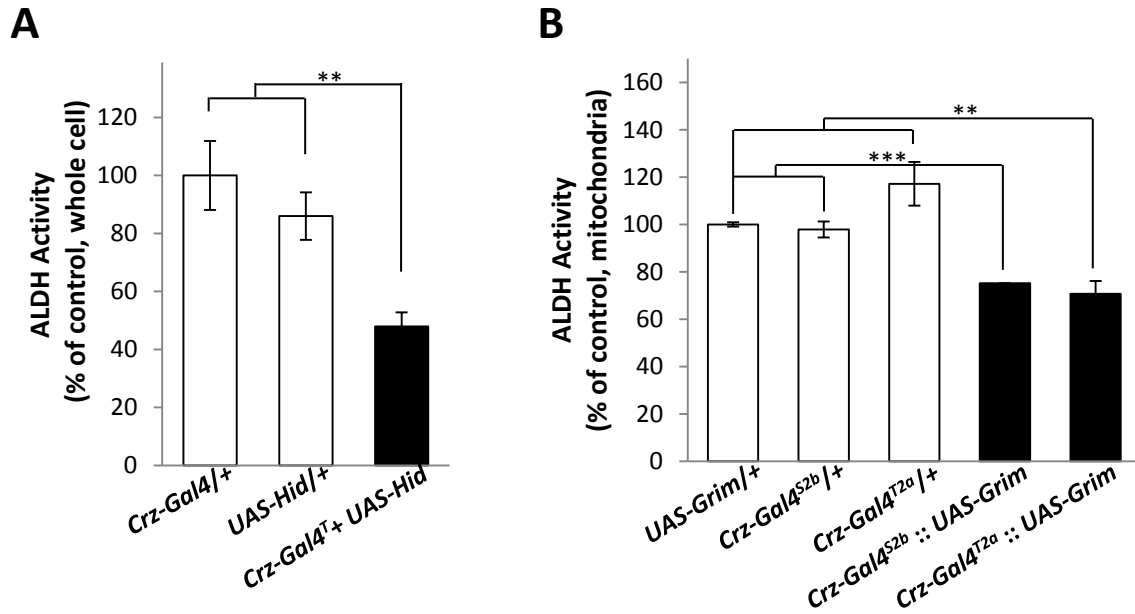


Figure 3-7. *Crz* cell ablation leads to reduced ALDH activity. (A) Whole cell ALDH activity of flies expressing pro-apoptotic gene *Hid* from *Crz-Gal4* reduced significantly, compared to *Crz-Gal4*^{+/+} and *UAS-hid*^{+/+} heterozygous controls (n = 8). **(B)** Expression of pro-apoptotic gene *grim* from *Crz-Gal4* drivers produced significant reduction of mitochondrial ALDH activity (n = 3). (***p* < 0.01, ****p* < 0.001). Each data point represents mean ± sem. All genotypes are in *y w* background.

that Crz signaling plays an important role for the regulation of ALDH activities for normal acetaldehyde oxidation.

IV-4. Loss of CrzR results in enhanced ADH activity

Since lack of *CrzR* reduces ALDH activity, *CrzR⁰¹* flies could be more sensitive to exogenously provided acetaldehyde. To test this, flies were fed with acetaldehyde. However, the mutant flies showed higher insensitivity to 0.5% and 1% of acetaldehyde than the *wild-type* (Fig. 3-8A). Around 40% enhanced survivability were observed from *CrzR⁰¹* flies compared to that of the *wild-type* after 60 h of 0.5 % acetaldehyde feeding. Similar results were also observed from flies subjected to 60 h of 1% acetaldehyde, with around 65% difference in survivability even after 30 h feeding. These results suggest a better detoxification of acetaldehyde by *CrzR⁰¹* flies, which could possibly be caused by higher ADH activities that transform acetaldehyde into ethanol in the absence of *CrzR*. If that is the case, one would expect to reduce such difference in survivability by inhibiting ADH activities in both genotypes. In order to inhibit ADH activity *in vivo*, both *wild-type* and mutant flies were pre-fed with 0.5% acetone (Anderson and McDonald, 1981; Barbancho, 1992). This pre-treatment significantly reduced survival of both genotypes, and almost removed the difference in sensitivity to acetaldehyde between *CrzR⁰¹* and *wild-type* flies (Fig. 3-8B). This result suggested an alteration of ADH activity in the mutant flies.

Next, we fed flies with sucrose containing 10-14% ethanol to investigate whether intoxication through feeding is altered in the *CrzR⁰¹* mutant. In wild-type, most flies were sober after four hours of feeding in all three ethanol concentrations. After this and onward, flies with 12% and 14% ethanol were rapidly knocked out after 8.5h, leaving 24% (with 12% ethanol) and 6.5% (with 14% ethanol), respectively, remaining sober (Fig. 3-9A). Surprisingly, 90% of flies were still resistant to 10% ethanol-induced knockout, indicating that this concentration is the threshold at which flies can detoxify. Remarkably, *CrzR⁰¹ mutant flies showed much stronger resistance* than did wild-type in all

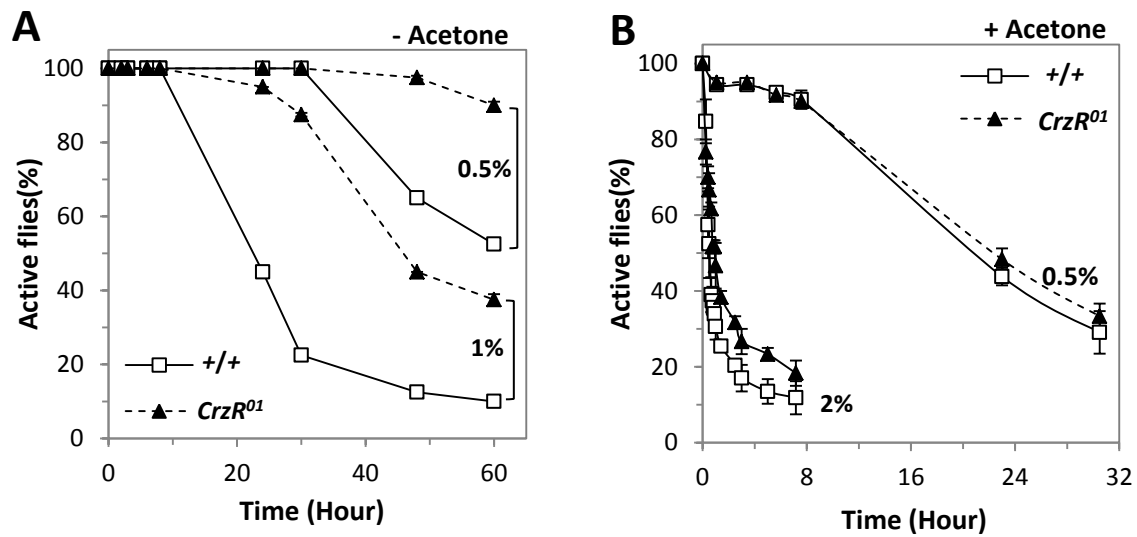


Figure 3-8. *CrzR* is required for better detoxification of acetaldehyde. Adult male lethality to acetaldehyde was tested on *CrzR* null (dashed lines) and *wild-type* (solid line) flies with none (**A**) or acetone pre-fed (**B**) treatment. Different acetaldehyde concentrations were applied as shown in the graphs ($n = 3$). Each data point represents mean $\pm$ sem.

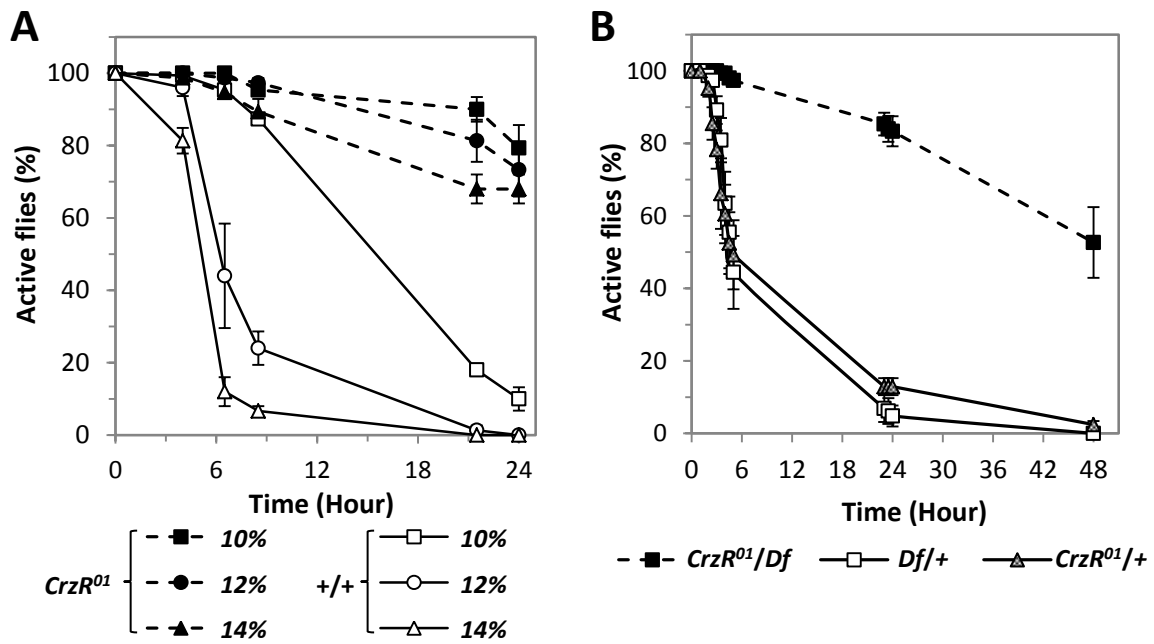


Figure 3-9. *CrzR* is required for better detoxification of ethanol. (A) Ethanol resistance of *CrzR null* (dashed lines) and control (solid line) flies was tested with concentrations ranging from 10% to 14% (n = 6). (B) Lethality to 14% ethanol was tested on *CrzR null* over corresponding deficiency, with *Df/+* and *CrzR⁰¹/+* as controls (n = 6). Each data point represents mean ± sem.

three ethanol concentrations. Around 80%, 73% and 68% of the mutant flies survived after 24-h feeding on 10%, 12% and 14% ethanol, respectively (Fig. 3-9A). Flies hemizygous for *CrzR*⁰¹ (*CrzR*⁰¹/*Df*) also showed significantly greater resistance to 14% ethanol, as compared to *Df*/+ and *CrzR*⁰¹/+ controls (Fig. 3-9B). Together these results suggested an alteration of ADH activity in the mutant flies.

Since ADH is known to mediate the detoxification of the acetaldehyde through the reverse reaction from acetaldehyde to ethanol in flies (Leal and Barbancho, 1992), we wondered if greater resistance to acetaldehyde by *CrzR*⁰¹ is due to higher ADH activity in the mutant. Intriguingly, around a 2.4-fold increase of ADH activity was observed in both homozygous *CrzR*⁰¹ and *CrzR*⁰¹/*Df* as compared to wild-type flies (Fig. 3-10A). *Df*/+ flies showed a 1.5-fold increase of ADH activity, suggesting a dosage dependent ADH regulation by *CrzR*.

IV-5. *CrzR* regulates *Adh* transcription in PKA-dependent manner

To further understand how *Crz*/*CrzR* regulates ADH and ALDH activities, mRNA levels of *Adh* and *Aldh* were analyzed via *q*PCR. Two distinct sets of primers were used for each gene to ascertain the product from the same gene transcript. An average of around 2.7-fold increase of *Adh* transcript levels was observed from *CrzR*⁰¹ allele using both primer sets (Fig. 3-10B), while no difference was found for *Aldh* (Fig. 3-10C). RT-PCR analysis also showed similar levels of *Aldh* transcript expression from *Crz*-cell ablated flies as the ones from wild-type controls (Fig. 3-10D) (Choi, 2009). These results indicate a role of *CrzR* in regulating the transcription of *Adh*, but not *Aldh*, in the adult flies.

To further understand signaling mechanisms underlying *CrzR*-regulated *Adh* transcription, we attempted transgenic manipulations in *CrzR*-cells. To do this, we first generated *CrzR-gal4* lines, as described in the method. Then these lines were used to drive expression of membrane-bound GFP (*mCD8GFP*) and nuclear RFP (*Redstinger*) simultaneously to visualize expression patterns. As

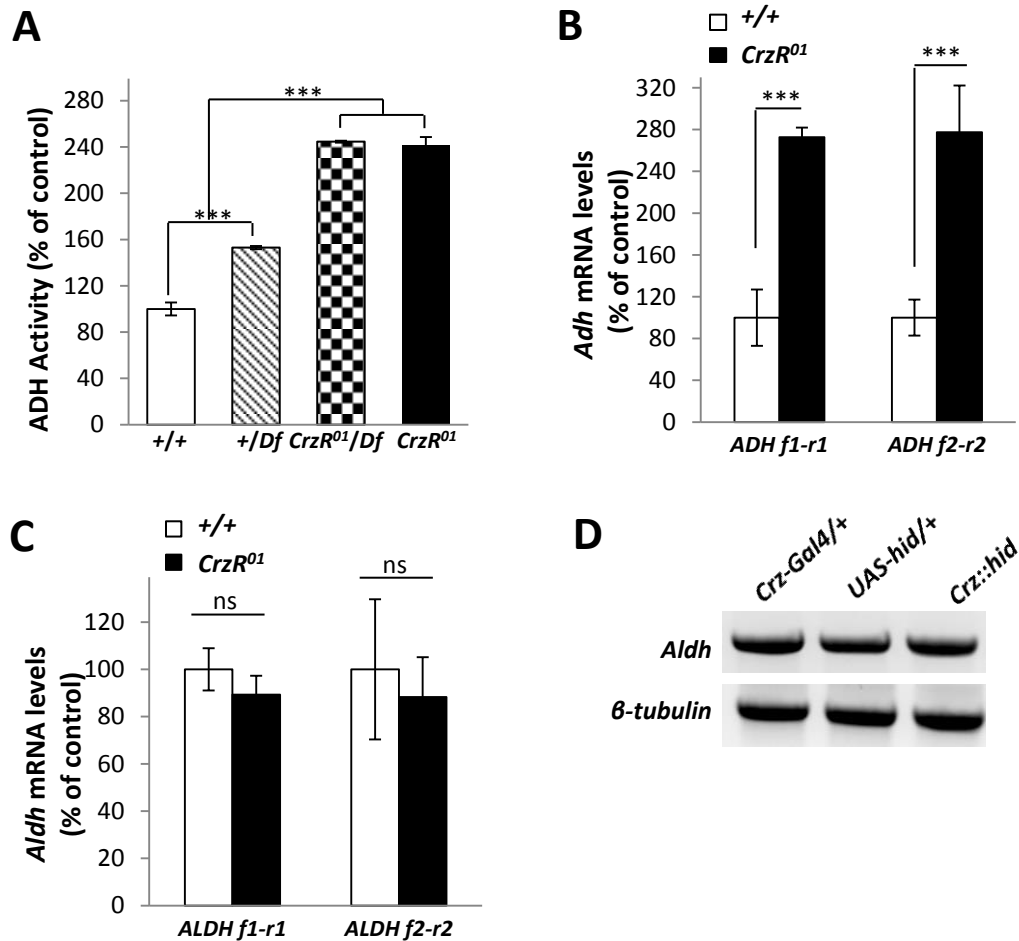


Figure 3-10. Lack of *CrzR* causes significant increase of ADH activity. (A) A histogram showing cytosolic ADH activity (n = 4, one-way ANOVA, SNK post-test). (B) Increase of *CrzR* transcript levels in the *CrzR*⁰¹ mutant. Quantitative RT-PCR using two different sets of primers revealed 2.7-fold increase of *Adh* transcript level in *CrzR*⁰¹ flies as compared to the wild-type (n=4, student t-test, paired two-tailed test). (C) No significant change of *Aldh* transcript levels was observed from *CrzR*⁰¹ flies ($P > 0.05$, n=4). (D) RT-PCR analysis *Aldh* transcript expression. Flies with *Crz* neuron ablation from *hid* expression did not change *Aldh* transcript levels.

shown in Figure 3-11, *CrzR-gal4* lines, florescent signals were detected in the larval CNS and salivary glands, while no expression was observed in the larval fat body (Fig. 3-11A-C). In the adults, strong GFP/RFP signals were detected in the fat body (Fig. 3-11D). These spatial and developmental expression patterns are consistent with the ones from high-throughput analysis (Flybase: flybase.org). Three independent lines showed consistent reporter gene expression patterns.

CrzR is a member of Class-A GPCR (Sha et al., 2012). A major signaling pathway of these receptors involves cAMP-dependent protein kinase, which regulates activity of a transcription factor CREB (cAMP response element binding protein) via phosphorylation. Thus we explored whether this is true for the CrzR-regulated expression of *Adh*. To do this, the PKA pathway was disrupted by ectopic expression of PKA inhibitor (*PKAⁱⁿ*), a dominant-negative form of the regulatory subunit of a *Drosophila* PKA (Rodan et al., 2002), or Cbz, a dominant negative CREB using CrzR-gal4 (Eresh et al., 1997). Remarkably, these flies displayed severe hangover phenotype that is similar to what was shown for the *CrzR⁰¹* mutant (Fig. 3-12A, B vs. Fig. 3-1A). Furthermore, ADH activity and *Adh* mRNA levels were significantly higher in CrzR::PKAⁱⁿ flies compared to the transgenic controls CrzR-gal4/+ and PKAⁱⁿ/+ (Fig. 3-12C, D). Like in the *CrzR⁰¹* mutant, neither ALDH activity nor *Aldh* mRNA expression was affected in the PKA-inhibited flies (data not shown). Together, these results suggest that CrzR-regulated *Adh* expression requires the PKA-dependent pathway. No positive role of protein kinase C (PKC) pathway was observed, as ectopic expression of PKC inhibitor showed normal pattern of hangover and ADH activity (Fig. 3-13).

In contrast to these foregoing results, Crz-cell deficiency did not cause higher ADH activity compared to the transgenic controls (Fig. 3-14). In other words, while CrzR is associated with the regulation of both ALDH and ADH activities, *Crz* neurons are required only for the regulation of ALDH activity. It is currently unknown the cause of such difference between the two genotypes. One possibility is that CrzR responds to an unknown ligand that leads to activate differential signaling pathways (Fig. 3-15) (see discussion).

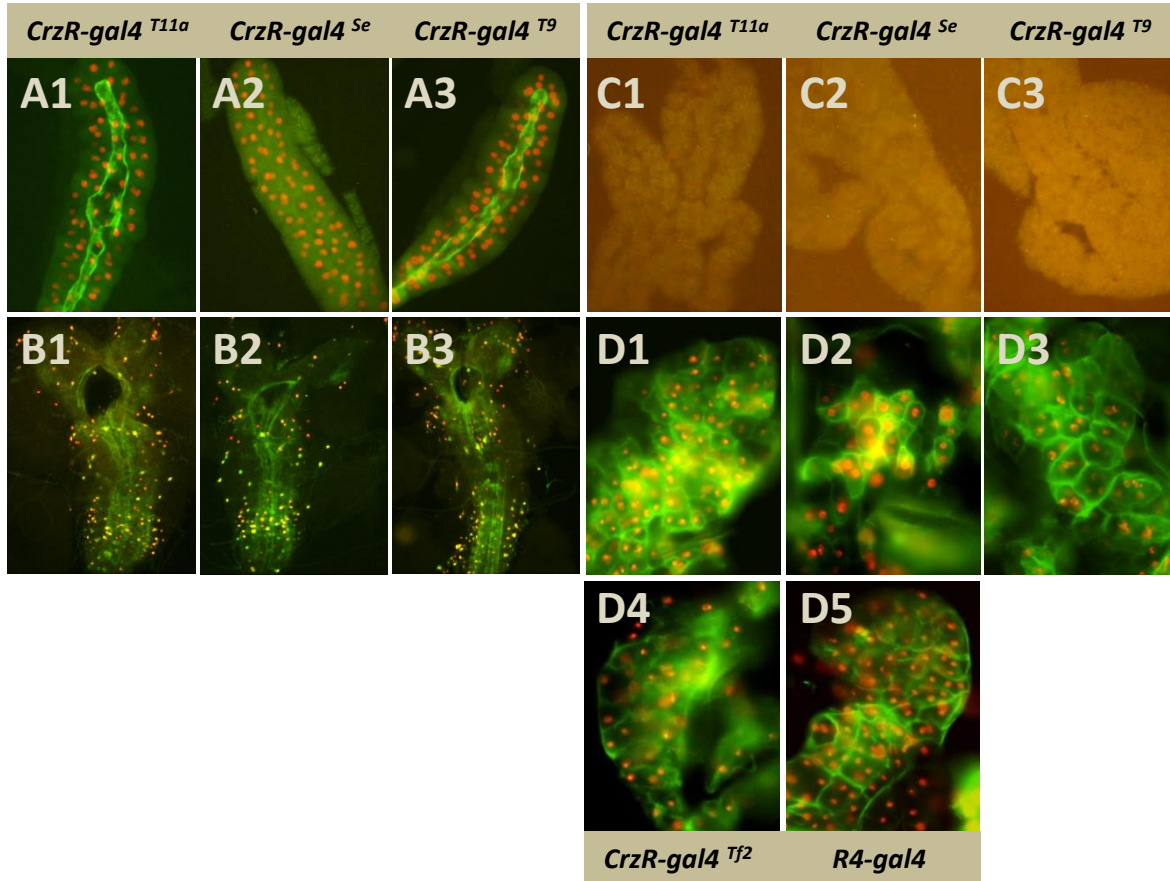


Figure 3-11. *CrzR-gal4* driven expression in larva and adult tissues. *UAS-mCD8GFP*; *UAS-Redstinger* was expressed under the control of *CrzR-gal4* drivers. **(A)** Third instar larval salivary gland. **(B)** Third instar larva CNS. **(C)** Third instar larval fat body. **(D)** Adult fat tissue. (Green: membrane bonded GFP, red: nuclear localized RFP).

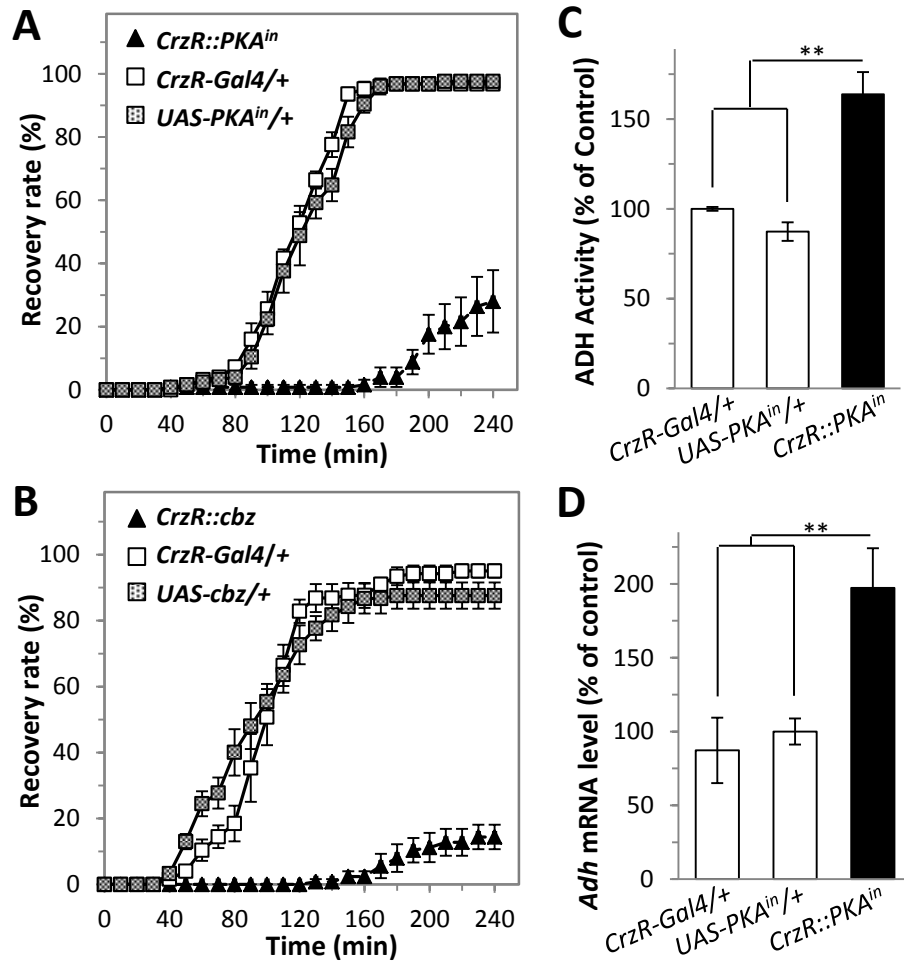


Figure 3-12. CrzR regulates *Adh* mRNA levels through PKA-dependent pathway. (A) Expression of PKA inhibitor by using *CrzR-gal4* induced severe hangover phenotype (n = 5). (B) Expression of a dominant negative form of CREB in CrzR cells caused severe hangover phenotype (n = 5). (C) PKA inhibition in CrzR cells produced 50% increase of ADH activity compared to transgenic controls (n = 3). (D) PKA inhibition in CrzR cells leads to significant increase of *Adh* transcript levels (n=3) (***P* < 0.005). Each data point represents mean $\pm$ sem. All genotypes are in *y w* background.

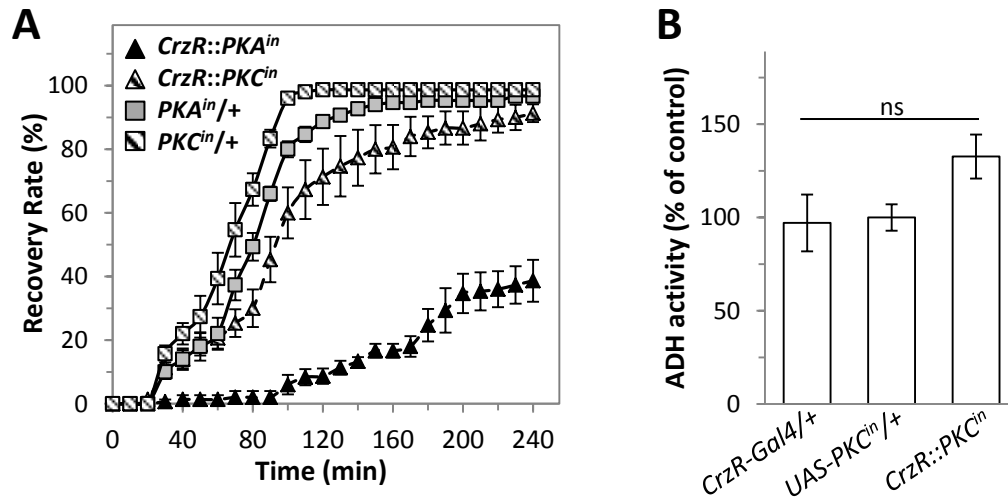


Figure 3-13. PKC has no regulatory effect on ADH. (A) No delayed recovery was observed from flies expressing PKC inhibitor from *CrzR-Gal4* ($n = 5$). Each data point represents mean $\pm$ sem. (B) Inhibition of *PKC* did not affect ADH activity ($P > 0.05$, $n = 3$). All genotypes are in *y w* background.

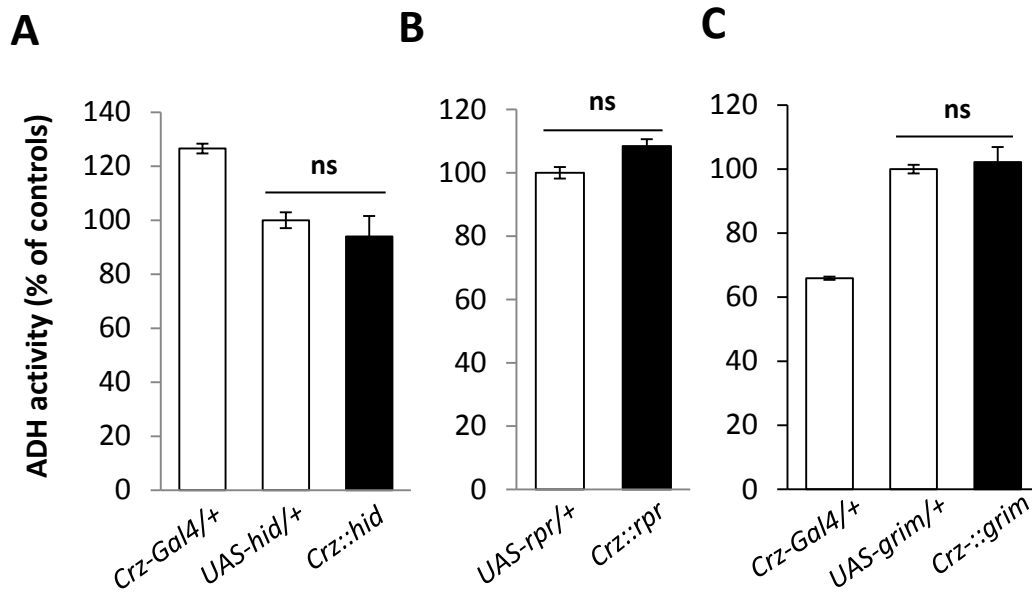


Figure 3-14. ALDH activity is not affected by lacking of *Crz* neuron. (A) *hid*- and (B) *rpr*-induced *Crz* cell ablation did not increase whole cell ADH activity. (C) No significant change of mitochondrial ALDH activity was observed from *grim*-induced *Crz* neuron ablation. ($P > 0.05$, $n = 3$). Each data point represents mean $\pm$ sem.

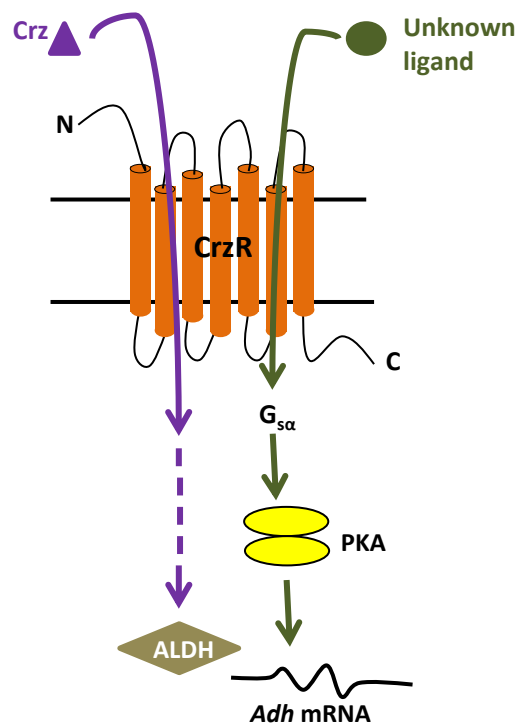


Figure 3-15. Model for differential activation of the CrzR. Crz and the unknown ligand exhibit different G-protein specificity thus triggering different signal transduction pathways. PKA, protein kinase A.

V. Discussion

Drinking ethanol is one of the oldest habits of humankind and has benefits to human society, but over-consumption of alcoholic beverages causes serious psychopathic symptoms and other health problems. Excessive alcoholic consumption and associated hangovers cost \$223.5 billion per year in the U.S. alone, due to losses in workplace productivity, health care expenses, costs associated with alcohol-related law enforcement and criminal justice, and vehicular accidents (<http://www.cdc.gov/Features/AlcoholConsumption/>).

Effects of ethanol consumption in humans include euphoria at lower blood alcohol concentration, impaired motor function and speech at moderate concentrations, followed by vomiting, coma, and even death in certain cases. A hangover is characterized by unpleasant physical pains such as headaches, sensory problems such as vertigo, gastrointestinal symptoms such as nausea and vomiting, disruption of sleep and biological rhythms, etc. (Swift and Davidson, 1998). The hangover is a result from acetaldehyde accumulation, as supported by high incidence of ethanol intoxication in Eastern Asian populations due to polymorphic deficiency of functional *ALDH2* allele (Crabb et al., 1989; Yoshida et al., 1984) and *ALDH2*-knockout mice (Isse et al., 2002). In line with this, drugs inhibiting ALDH, such as disulfiram, have been used to treat chronic alcoholism by reducing symptoms from ethanol intake (Chick et al., 1992; Wright and Moore, 1990). Despite these studies, little is known about how physiological factors regulate ethanol metabolism.

Fruit flies feed on fermenting fruits with varying alcohol contents. Like mammals, *D. melanogaster* metabolize ethanol to acetaldehyde and acetate catalyzed by ADH and ALDH, respectively (Gibson et al., 1981), and *Adh* and *Aldh* mutant flies showed dramatically reduced tolerance to ethanol (Fry and Saweikis, 2006). Intriguingly, our present data suggests that a neuroendocrine system plays an important role in ethanol metabolism, as mutant flies lacking Crz neurons or CrzR showed severely impaired recovery from ethanol-induced sedation. By comparison, several neuropeptides have been shown to control the sensitivity to ethanol-induced sedation, as

mentioned earlier. These studies together suggest that the peptidergic nervous system is intricately associated with ethanol-related tolerance.

Our data indicate that CrzR activation leads to the transcriptional repression of *Adh* via the PKA-dependent pathway and to post-transcriptional activation of ALDH via unknown pathways. Recently, CrzR isolated from the silkworm, *Bombyx mori*, was shown to couple dually to the Gq and Gs proteins in cell-based assays (Yang et al., 2013). However, results from the expression of transgenic inhibitors indicate that Gq protein-associated PKC activation is unlikely to be involved in the regulation of *Adh* transcription in *Drosophila*. Thus we propose that Gs-led PKA activation is the major *in vivo* signaling pathway of the CrzR in *Drosophila*.

In contrast to *CrzR⁰¹*, Crz-CD did not affect ADH activity levels, but reduced ALDH activity. Such a difference between Crz-CD and *CrzR⁰¹* could explain how *CrzR⁰¹* flies displayed a more severe hangover phenotype that did Crz-ablated flies. From these results, we propose that CrzR mediates two separate signaling pathways: Crz-dependent up-regulation of ALDH activity and Crz-independent down-regulation of *Adh* transcription via PKA activation (Fig. 3-15). The latter pathway might involve an additional ligand that activates CrzR. One possible candidate is adipokinetic hormone (AKH), as the AKH receptor is structurally related to the CrzR with 56% amino acid sequence similarity (Cazzamali et al., 2002). However, CrzR showed very little affinity for AKH (Park et al., 2002a), indicating that AKH is not a natural ligand for CrzR.

Although the identity of the second ligand for CrzR is speculative, a growing body of evidence suggests that a GPCR can be coupled to different G-proteins in response to different agonists; such molecular flexibility is often referred to as “functional selectivity” of GPCR (Deupi and Kobilka, 2007; Seifert and Dove, 2009). According to this hypothesis, binding properties of different ligands induce and stabilize a unique conformational status of GPCR, which in turn shifts coupling preference to different G-proteins. As mentioned earlier, *Bombyx* CrzR can couple to Gs and Gq proteins (Yang et al., 2013). These studies suggest that CrzR is an excellent model system to unravel the physiological dynamics of the GPCR.

The International Agency for Research on Cancer (IARC) classified alcohol as a Group 1 carcinogen (<http://monographs.iarc.fr/ENG/Classification/ClassificationsAlphaOrder.pdf>). Accumulation of acetaldehyde as a result of defective ALDH2 is a major culprit of carcinogenesis (Boccia et al., 2009). In line with this, many cancer cells have disproportional activities of ADH and ALDH, making them less efficient in removing acetaldehyde compared to normal tissues (Jelski and Szmitkowski, 2008). In this regard, *CrzR⁰¹* flies might be prone to cancer development, and perhaps can serve as an interesting model system to elucidate the mechanisms of ethanol-induced carcinogenesis.

Chapter 4

Characterization of CrzR from the House Fly, *Musca domestica*

This chapter is revised based on paper publication by Kai Sha and W. Craig Conner

Sha, K., Conner, W.C., Choi, D.Y., Park, J.H., 2012. Characterization, expression, and evolutionary aspects of Corazonin neuropeptide and its receptor from the House Fly, *Musca domestica* (Diptera: Muscidae). *Gene* 497, 191-199.

My primary contribution to this paper include a) development of a problem into a work, b) design and conducting the experiments, c) collecting and analyzing data, d) most of the writing.

I. Abstract

To gain the evolutionary aspect of *CrzR*, *CrzR* from the House Fly, *Musca domestica* (*MdCrzR*) was cloned and characterized. *MdCrzR* deduced from the full-length cDNA sequence is a 655-amino acid polypeptide that contains seven *trans*-membrane (TM) domains and other motifs that are characteristic of Class-A G-protein coupled receptors. Although the TMs and loops between the TMs are conserved in other *CrzRs*, the *N*-terminal extracellular domain is quite dissimilar. Tissue-specific RT-PCR revealed a high level of *MdCrzR* expression in the larval salivary glands and a moderate level in the CNS. In adults, the expression was broadly observed without significant gender difference, suggesting multifunctionality of the *Crz* signaling system.

II. Introduction

CrzR is a member of Class-A GPCR that share several structural features among diverse species. (Hofmann et al., 2009; Rosenbaum et al., 2009). These features include the E/DRY motif that is involved in agonist binding, receptor structure and activation process (Fraser et al., 1988), the CWxP motif serving as a rotamer toggle switch (Hofmann et al., 2009; Shi et al., 2002) and the NPxxY motif (Fritze et al., 2003) that is important for the formation of the TM1-TM2-TM7 network (Hofmann et al., 2009). The disulfide bond (C255, C291) that links the first and second ELs is also a feature shared in all Class-A GPCRs (Hausdorff et al., 1990). In some GPCRs, palmitoylated Cys

residues in the cytoplasmic tail anchor the receptor to the plasma membrane, forming extra cytoplasmic loops (Chini and Parenti, 2009).

High-throughput analysis revealed the salivary glands as a major and CNS as a minor tissue that express *CrzR* in *D. melanogaster* (Flybase: flybase.org). In adults, the high-throughput analysis shows a high level of *CrzR* expression in the fat body, heart, and epidermis; intermediate levels in the salivary glands; and low levels in the CNS (Flybase: flybase.org). These data suggest multifunctionality (neuronal and non-neuronal roles) of the Crz particularly in adults.

To gain insight into the evolutionary prospects of Crz-associated biology in a fly species distantly related to *D. melanogaster*, we isolated and characterized the genes for CrzR in the House Fly, *Musca domestica*. This species is a medically important pest, transmitting a number of microbial pathogens, and has thus been a target of pest-control research (Scott et al., 2009). Having diverged from *Drosophila* lineage (Section Acalyptrate) around 100 million years ago (Beverley and Wilson, 1984), *M. domestica* (Section Calyptrate) has evolved a unique genetic makeup, providing the bases for distinct behavioral patterns, habitats, lifestyle, physiology, anatomy, and development. Such divergence makes *M. domestica* a well-placed phylogenetic intermediate to relate *Drosophila* Crz studies to those of more remotely related dipterans, such as mosquitoes.

III. Materials and methods

House Fly

The House Fly larvae and pupae were obtained from Carolina Biology (www.carolina.com) and incubated at room temperature. Enclosed adults were collected and maintained in a 1000-ml jar containing a food (one to one mix of dry milk and sugar) and water. The flies were also provided with larval food (Carolina Biology) for egg laying and larval growth in a milk bottle.

Cloning of *MdCrz* receptor (*MdCrzR*)

The cloning strategy for the *MdCrzR* cDNA is illustrated in Figure 4-1. Partial gene sequence encoding *MdCrzR* was first cloned by PCR using degenerate primers that were derived from the conserved domains among three known CrzRs (Table 4-1). PCR reactions (25 µl) included 12.5 µl 2X GoTaq Green PCR Master Mix, 1 µl of cDNA and 8 pmole of each primer (PCR parameters: 94° for 2 min, 30 cycles of 94° for 40 sec 53° for 40 sec 72° for 1 min, and final elongation at 72° for 4 min). The first PCR performed with dgCrzR-f1/dgCrzR-r2 primers yielded no visible product. The reaction was then diluted 1:50 in water and used as a template for the nested-PCR using dgCrzRf2/ dgCrzR-r1 primers. This PCR produced a ~400-bp fragment that we confirmed to be specific to *MdCrzR*. According to this sequence, gene-specific reverse primers (MdCrz-r1 and MdCrz-r2), along with degenerate forward primers (dgCrzR-f3 and dgCrzR-f4), were used for PCRs, which resulted in an additional ~320-bp product upstream of the previous 400-bp sequence. To obtain the full-length *MdCrzR* cDNA sequence, we undertook RACE using newly designed *MdCrzR*-specific primers. PCRs were performed in a manner similar to *MdCrz* 3'-RACE. The first PCR was done with MdCrzR-3RACE-f1/Ad-I primers and then the nested PCR with MdCrzR-3RACE-f2/Ad- II primers. PCR products (~1 kb) were then gel-purified, inserted into pGEM-Teasy vector, and sequenced. For 5'-RACE, cDNA was first produced by a reverse transcription reaction with MdCrzR- 5RACE-r2 primer, and then poly-A tailed, as described previously. Two consecutive PCRs were performed with two sets of primers: MdCrzR-5RACE-r2/oligo-dT-Ad and MdCrzR-5RACE-r1/Ad-I. Approximately 1-kb PCR product was obtained, cloned into pGEM-Teasy vector and sequenced.

RT-PCR analysis for tissue-specific expression.

Approximately 80 0-2-day-old adult flies of one gender were frozen and decapitated by vigorously shaking the tubes. Subsequently, the heads were separated from the body parts with no. 25 and no. 40 brass sieves. Separated tissues were homogenized in 500 µl of ice-cold Trizol reagent

Table 4-1. PCR Primers used for analyzing MdCrzR.

Name	Direction	Sequence
dgCrzR-f1	for	TTYGTIGARGARTTYTAYCA
dgCrzR-f2	for	TAYCARTGYGTIACICAYGG
dgCrzR-f3	for	GAYGTIYTIGTIACITGGTT
dgCrzR-f4	for	TGGTTYTGYATHATHGGIGARGC
dgCrzR-r1	rev	TTISWCATICCRAARAARAADAT
dgCrzR-r2	rev	ARRTGRAAIGCICCRTADAT
MdCrzR-r1	rev	GTAAGGCGCATGCGTCACACACTG
MdCrzR-r2	rev	CCTGCCATTCGGCGGTGTAAAAG
MdCrzR-f1	for	GAAGCTGTTTCAAATGTTTACGCTG
MdCrzR-3RACE-f1	for	GCTGGACGCCGTACAATGTCATGATG
MdCrzR-3RACE-f2	for	GATATTCATGTTTTGGAATCCGGAC
MdCrzR-5RACE-r1	rev	CCAGCTTGCAAGTGAGCTCATTGG
MdCrzR-5RACE-r2	rev	GAGCCTTGGAACATTTTTTCAC
Md-GAPDH-f1	for	CAAGGGTGGTCCCAAGAAGGTC
Md-GAPDH-r1	rev	CAGATACATTGGGAGTGGGTACACGG

R = A, G; Y = C, T; S = C, G; W = A, T; H = A, C, T; D = A, G, T; I = A, C, G, T

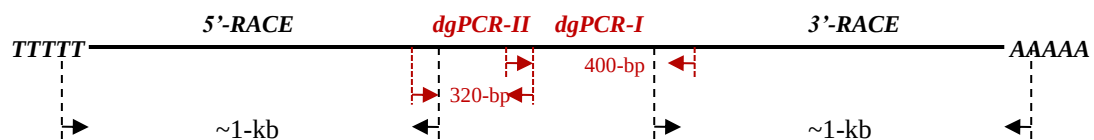


Figure 4-1. Schematic illustration of the cloning strategy of the *MdCrzR*. Regions amplified by PCRs using degenerate primers are indicated as dgPCR-I and -II.

(Invitrogen), and total RNA was extracted following recommended procedures. Typically 200-500 ng of RNA and 10 pmole of primers were included in a 50 µl Superscript RT-PCR kit (Invitrogen). f2 and r1 primers were used for *MdCrz*, and MdCrzR-f1/MdCrzR-5RACE-r2 primers for *MdCrzR*. Control reactions were run in parallel with the primers for the House Fly *GAPDH* gene (Table 4-1). Each reaction was performed three times with similar results.

IV. Results

IV-1. Cloning and characterization of *MdCrzR*

cDNA sequence is available in the GenBank database (accession no. JN003596; see also Fig. 4-2). The *MdCrzR* cDNA is composed of 1968-bp ORF encoding putative 655-amino acids (aa) MdCrzR, 228-bp 5'-UTR and 204-bp 3'-UTR. As expected, web-based software (SOSUI: <http://bp.nuap.nagoya-u.ac.jp/sosui/>) predicted this protein as a G-protein coupled receptor (GPCR) containing seven *trans*-membrane (TM) helices, N-terminal extracellular domain (ECD), three extracellular loops (ELs), three intracellular loops (ILs), and C-terminal intracellular domain (ICD) (Fig. 4-3). Based on comparative analysis, CrzRs belong to the Class-A rhodopsin-like GPCR family (Vadakkadath Meethal et al., 2006), which is by far the largest and most studied GPCRs. Class-A GPCRs share several structural features (Hofmann et al., 2009; Rosenbaum et al., 2009). R223 residue in the cytoplasmic end of the TM3, a typical part of E/DRY motif, is thought to be involved in agonist binding, receptor structure and activation process (Fraser et al., 1988). Variants of this motif are found in other GPCRs including MdCrzR (DRW). Although the E/DRY motif is also important for the formation of 'ionic lock' with an acidic residue (E) in the TM6, thereby stabilizing the receptor conformation in the inactive state (Vogel et al., 2008), such a residue is not found in the MdCrzR.

Another conserved motif is CWxP within the TM6, serving as a rotamer toggle switch, which is important for the receptor to attain active conformation upon ligand binding (Shi et al., 2002;

Figure 4-2. Nucleotide and deduced amino acid sequence of the *MdCrzR* cDNA. The translation start and termination codons are highlighted in gray and predicted amino acids are shown below the nucleotide sequence. Translation initiation site was determined based on the translation start consensus sequence found in *Drosophila* genes (C/A A A A/C ATG; Cavener, 1987). Seven predicted trans-membrane domains are boxed and labeled TM1–7. Motifs conserved in other GPCRs (DRW, CWxP, NPxxY, and ExxxLL) are indicated in red. The *N*-terminal region of the *MdCrzR* does not appear to have features characteristic of the signal peptide. Thus, it is most likely that *MdCrz* targets the plasma membrane through the TM1 domain, as is the case for the majority of GPCRs.

ACAAATCAGCGAGAATAAAACCAACAAAGCCCAAAGGTCAGCAATTGCATGTGGATGGGC
 GAGCTTCCGGCCACCAGTTTTACTAGACTCCTCTCAGATAATACGTTTCGGAATAAAAAGG
 CAACAAAACGAATCGGAATCGACTTCCTCTGCCTCGGGAGCATAAATTAATATAAATCAA
 AAATGCCAATTTCCAAAAGCAAAGTGTAACATTTCTGAGGCGACAAAATGAATCTGAAT
 M N L N **ECD**

ATCCTTACCCCTCAAATGGAACGGGTGCCGGCCATTGATGAGATAATTCATGTGGCCAGT
 I L T P Q M E R V P A I D E I I H V A S

GGCCTAACAAACGAACTTTACCCGCAATGTGTTTGGCATAGCCAATTCCACAATGAAACTA
 G L T T N F T R N V F G I A N S T M K L

TTAAATAGCCCTGCGAATGACACCAACTCCGCCACAATATCTCCACCCCTGTCAGACATG
 L N S P A N D T N S A T I S P P L S D M

AGTGATAATAGCGGCGATACAACGATAACCACGACCACCGCCCGTCACTGGCTACTGCAT
 S D N S G D T T I T T T A R H W L L H

GAGGGATATTTGGTCAACAGCAGCTATGCCTTTCTCACCAACCTGTCGTACACCAAACCC
 E G Y L V N S S Y A F L T N L S Y T K P

CATGTCTATGTCCCCGCCAGCACTCTGCCCCGCCATTGAAGATGACGAAATACGACTGGAA
 H V Y V P A S T L P A I E D D E I R L E

CATGCCCCTCAACTATCCAAATCGGCCAATGATTAAGGTTTATGTTTTAAGTTTGATGGCC
 H A P Q L S K S A M I K V Y V L S L M A **TM1**

CTCTTCTCACTGCTCGGCAATGTGCTGACCATGTGGAATATTTACAAGACCCGCATGGCT
 L F S L L G N V L T M W N I Y K T R M A **IL1**

CGACGCAATTCCCGTCACAGTTGGAGTGCCATCTATTTCGTTGATATTCCATCTCTCGATT
 R R N S R H S W S A I Y S L I F H L S I **TM2**

GCGGATGTTTTGGTAACTGGCTTTTGTATAATCGGTGAGGCGGCCTGGTGTACACCGTC
 A D V L V T G F C I I G E A A W C Y T V **EL1**

CAGTGGCTGGCCAATGAGCTCACCTGCAAGCTGGTGAAGCTGTTTCAAATGTTTCAGCCTG
 Q W L A N E L T C K L V K L F Q M F S L **TM3**

TATTTGTCCACTTATGTACTGGTCCTAATTGGCATAGATCGTTGGATAGCTGTCAAATAT
 Y L S T Y V L V L I G I **D R** **W** I A V K Y **IL2**

CCGATGAAGTCGCTGAACATGGCCAAGCGATGTTATCGTCTACTGGGTGGAACGTATATC
 P M K S L N M A K R C Y R L L G G T Y I **TM4**

CTGTCGTTTATCCTGAGTTTGCCACAGTTTTTCATATTTTCATGTGGCGCGTGGTCCGTTT
 L S F I L S L P Q F F I F H V A R G P F **EL2**

GTGGAAGAGTTCTATCAGTGTGTGACGCATGGCTTTTACACCGCCGAATGGCAGGAGCAG
 V E E F Y Q C V T H G F Y T A E W Q E Q

CTTTACGCAACATTTACTTTAGTTTTTACACTCCTGCTGCCGCTGTGTATACTCTTCGGA
 L Y A T F T L V F T L L L P L C I L F G **TM5**

AGCTACATGTCGACATTTTCGCACCATTTCAAGCAGTGAAAAAATGTTCCAAGGCTCAAAA
 S Y M S T F R T I S S S E K M F Q G S K **IL3**

TTGGCCGACTACACCAACAAACCTTCGAGCAAAACAAATCGCCAACGTCTGACACACAAG
 L A D Y T N K P S S K T N R Q R L T H K

GCGAAAAATGAAGTCACTGCGCATCTCGGTGGTCATCATATTGCCTTTCTCATCTGCTGG
 A K M K S L R I S V V I I I A F L I **C W** **TM6**

ACGCCGTACAATGTCATGATGTTGATATTCATGTTTTGGAATCCGGACAAGAGATTCGGT
T P Y N V M M L I F M F W N P D K R F G **EL3**

GAAGATTTACAATCGGCCATATTCTTTTTTGGCATGTCCAACAGTTTGGTCAATCCCCTC
 E D L Q S A I F F F G M S N S L V **N P L** **TM7**

ATCTACGGAGCCTTCATCTCTGTCCCATGAAACGGAAGACCAGCAAAAAGGGCAACAAC
I Y G A F H L C P M K R K T S K K G N N **ICD**

AACAAATGGCAACTATAGCCTCAACAGAGGTGACTCACAAAGGACCCCCTCCATGTTGACT
 N N G N Y S L N R G D S Q R T P S M L T

GCCGTGACACAAATTGATTGCAATGGCCGTCAGGTGAGAACATATCGCCAGACGAGTTAC
 A V T Q I D C N G R Q V R T Y R Q T S Y

TATCGCAGCATCAGTACGGGCAGCAATTACAAGGAGAATATTGGTCTACTGCAGACGCCC
 Y R S I S T G S N Y K **E N I G L L** Q T P

ACAAATAATGGTGGGGGCACACCCACACCCGGTCTAATACGGAAATCGGCCTCCCTGAGA
 T N N G G G T P T P G L I R K S A S L R
 AGTCCCCATCCGAACCAGAGATCATTGGTACACACTGCAGGTAGTAGACGCAATTTTAAT
 S P H P N Q R S L V H T A G S R R N F N
 CAACCGTTAAGAGCTGGCGTCATGGACGATTACGATAATCAGCGACAGTATCACAACCAT
 Q P L R A G V M D D Y D N Q R Q Y H N H
 TCCAGCGAAATAATCCTCAATGACAACCTCCAGCAATGACGCCGGTCCTGTTGTGGTCAGT
 S S E I I L N D N S S N D A G P V V V S
 GAAGGCCAAGCCGTCTCCATTGTGCTGAGCTATGACAACCAACGTGGAGGTGTGGCAGCA
 E G Q A V S I V L S Y D N Q R G G V A A
 ATGAAACGCTCCAATACAGGTTCCATAAGCCCAACGGCAGCACCCCTTCACCGCACCAAAG
 M K R S N T G S I S P T A A P F T A P K
 AATGGTTATCGAACAGCGAATAATCTGTCCAACACCCTAGGCATTGGTCCAGCCACCTAC
 N G Y R T A N N L S N T L G I G P A T Y
 GGCAATTGTGGCAACGACAGTGTATCGAGTGTATGAGACATAATGTATGGATGGAACCCG
 G N C G N D S V S S V end
 GAGGAGTTTTACATCACCCGACAACAATTAGAGACTGGTTTTAAAAACAAAATGACA
 AACAATTGTTTTCCAAAAACAAGTTGTAACCTAGCCACGATGACCACCACCAACAA
 CAAAACAGCGACAACATTTTTTTTCTGTAGCCAAGCGAAAAAAAAAACTTTAAAGTAAACC

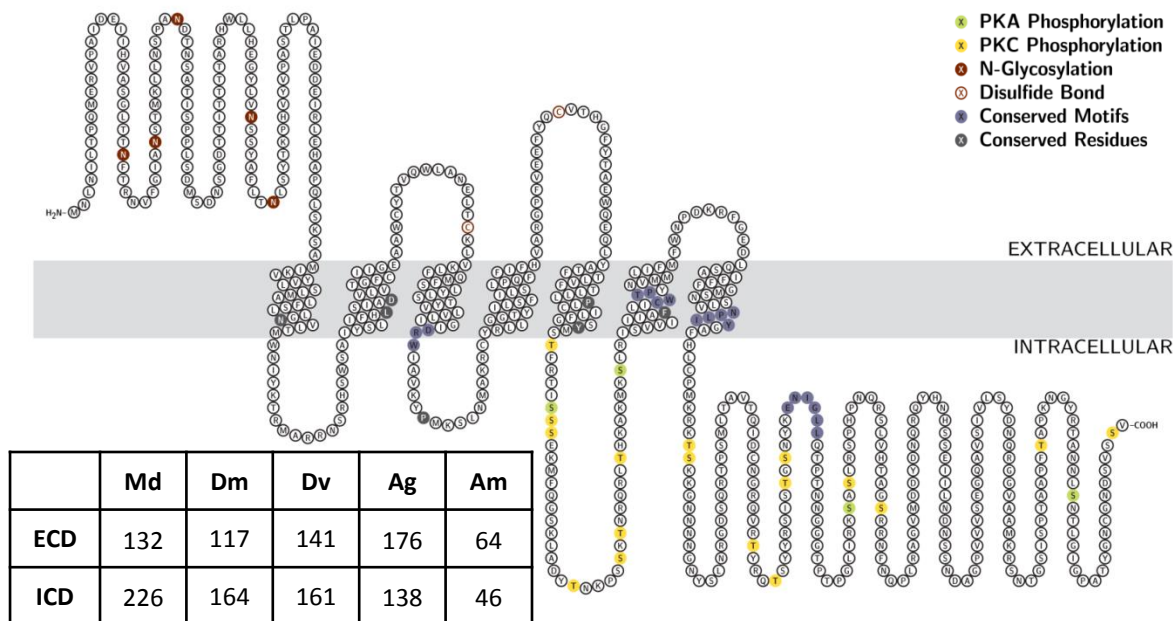


Figure 4-3. Schematic diagram of the MdCrzR structure predicted by a web-based software (SOSUI: <http://bp.nuap.nagoya-u.ac.jp/sosui/>). Seven helical *trans*-membrane domains (TM I-VII) are connected by three extracellular and three cytosolic loops. Box includes size comparison of the *N*-terminal extracellular domain (ECD) and C-terminal intracellular domain (ICD) of the homologous receptors in other insects (Dm, *D. melanogaster* (accession no. NP_648571); Dv, *D. virilis* (XP_002046915); Md, *M. domestica*; Ag, *An. Gambiae* (XP_321555); Am, *Ap. mellifera* (NP_001137393)). Putative post-translational modification sites and conserved motifs are also indicated, as follows. A pair of putative Cys residues that form S-S bond by red hollow circle (DiANNA 1.1: <http://bioinformatics.bc.edu/clotelab/DiANNA/>); putative *N*-glycosylation sites (NetNGlyc 1.0: <http://www.cbs.dtu.dk/services/NetNGlyc/>), PKA and PKC phosphorylation sites (NetPhosK 1.0: <http://www.cbs.dtu.dk/services/NetPhosK/>) by red, green and yellow, respectively. Conserved residues among class-A GPCRs in the TM regions are indicated in gray and other consensus motifs, DRY, CWxP, NPxxY, and ExxxLL, are highlighted in blue.

Hofmann et al., 2009). Near the cytoplasmic end of the TM7 helix, there is a conserved NPxxY motif (Fritze et al., 2003), which is important for the formation of the TM1-TM2-TM7 network (Hofmann et al., 2009). Upon receptor activation, these compact inter-helical structures undergo conformational changes to activate the signaling pathway.

MdCrzR also contains consensus Cys residues (C255, C291), possibly linking the first and second ELs through a disulfide bond, a feature shared in all Class-A GPCRs (Hausdorff et al., 1990). In some GPCRs, palmitoylated Cys residues in the cytoplasmic tail anchor the receptor to the plasma membrane, forming extra cytoplasmic loops (Chini and Parenti, 2009). There are two Cys in the ICD of the MdCrzR, but it is a matter of conjecture whether these residues are modified with lipid molecules. Putative post-translational modification sites (N-linked glycosylation, phosphorylation by PKC or PKA) that might be important for desensitization are indicated in Fig. 4-3.

Precise intracellular trafficking of the GPCRs to the plasma membrane is critical for their function. The first step in trafficking is to enter the endoplasmic reticulum (ER) where the nascent polypeptides are properly folded into the membrane. Two distinct features of the nascent GPCR can target it to the ER: the first is a signal peptide at the N-terminus that is usually cleaved by signal peptidase. The second one is a non-cleavable 'signal anchor' in the first TM domain (Higy et al., 2004). Although a minority of GPCRs has both motifs for ER entry (e.g., (Kochl et al., 2002), the majority has only the TM1-associated signal anchor sequence. Bioinformatics analysis of the MdCrzR done by SignalP (www.cbs.dtu.dk/services/SignalP/) also did not reveal a cleavable N-terminal signal peptide, indicating that TM1 is sufficient for MdCrzR membrane anchoring.

Appropriately folded GPCRs must be exported from the ER and traffic through the *trans*-Golgi network (TGN) to reach the plasma membrane. The export from the ER is considered the rate-limiting step and involves several export signals found in various GPCRs (Dong et al., 2007). In the MdCrzR, we found a consensus ExxxLL motif (ENIGLL) as a putative ER export signal in the cytoplasmic tail (Fig. 4-4A).

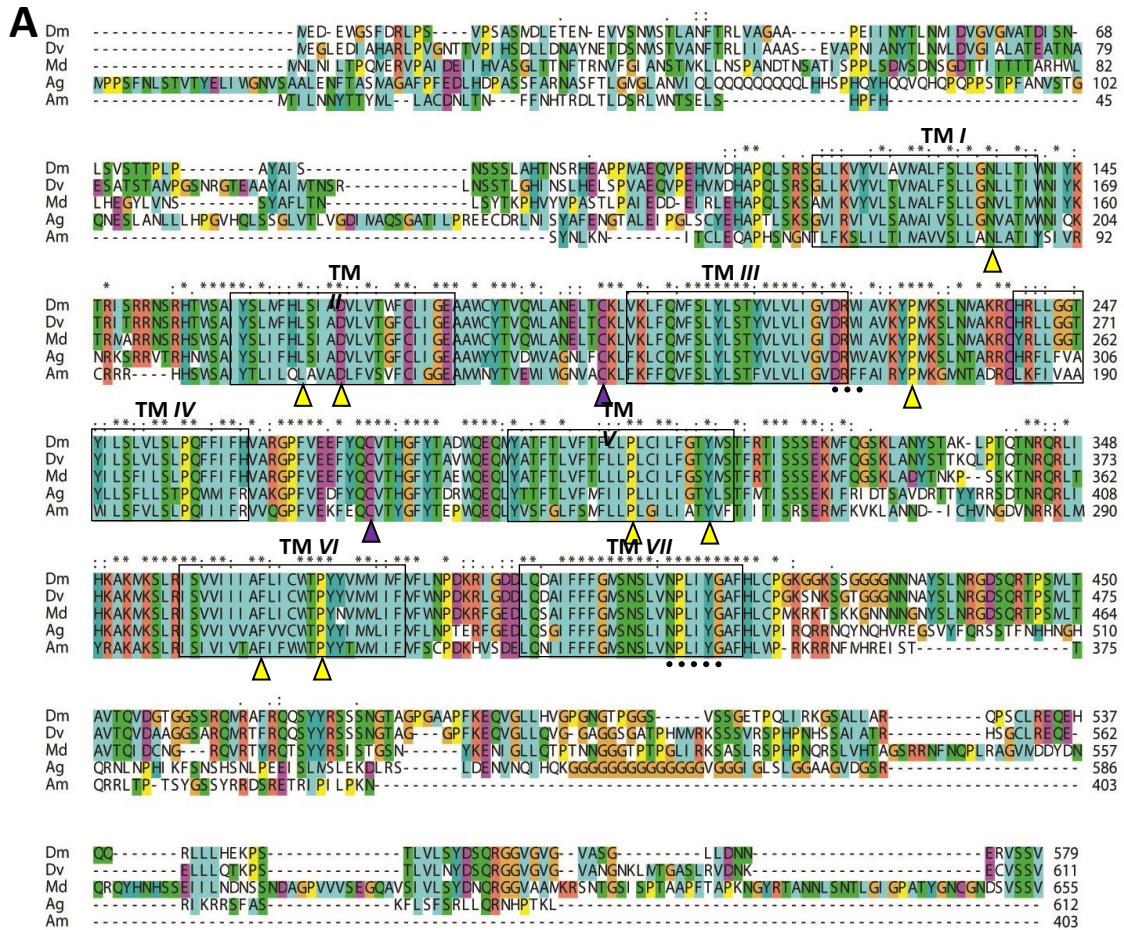


Figure 4-4. Characterization of the MdCrz receptor (MdCrzR). (A) Alignment of the CrzRs. The color codes are described in the following website: http://ekhidna.biocenter.helsinki.fi/pfam2/clustal_colors. (B) Domain-specific homology scores between Md and other sequences, as determined by ClustalW. (ECD, extracellular domain; ICD, intracellular domain).

During GPCR evolution, the seven TMs' core structure remained highly conserved. In contrast, regions related to binding and responding to ligands have diverged in size and chemical and physical properties (Rompler et al., 2007). Multiple sequence analysis also shows strong conservation in TM domains and loop regions among distantly related CrzRs (Fig. 4-4 A, B). Of interest, however, CrzRs have variable ECD (65-177 aa) and ICD (65-188 aa) sizes (Fig. 4-3). The ICD of MdCrzR shows ~ 40% identity to *Drosophila* species, whereas there is virtually no identity to *An. gambiae* or *Ap. mellifera*. The ECDs exhibit even more divergence, sharing only 7~21% identity between MdCrzR and its homologs. Thus it will be interesting to determine whether the ECD region is important for binding specificity and/or affinity to the ligand, and whether divergent ICDs mediate different modes of signaling transduction.

IV-2. Expression of the *MdCrzR*

To examine tissue-specific expression of *MdCrzR*, RT-PCR was performed. In the larvae, we found the salivary glands as a major expression site, while the CNS expresses *MdCrzR* weakly (Fig. 4-5A). Consistent with this pattern, high-throughput analysis revealed the salivary glands as a major and CNS as a minor tissue that express *CrzR* in *D. melanogaster* (Flybase: flybase.org). In adults, we found expression of *MdCrzR* both in the heads and bodies. No obvious gender difference was observed in the level of expression (Fig. 4-5B). Such observation is consistent with the high-throughput analysis of *D. melanogaster* adults, as it shows a high level of *CrzR* expression in the fat body, heart, and epidermis, intermediate levels in the salivary glands, and low levels in the CNS (Flybase: flybase.org).

V. Discussion

The conserved residues in GPCR have been reported to have significant effect on ligand binding or G-protein coupling selectivity. In our study, multiple sequence analysis also show strong

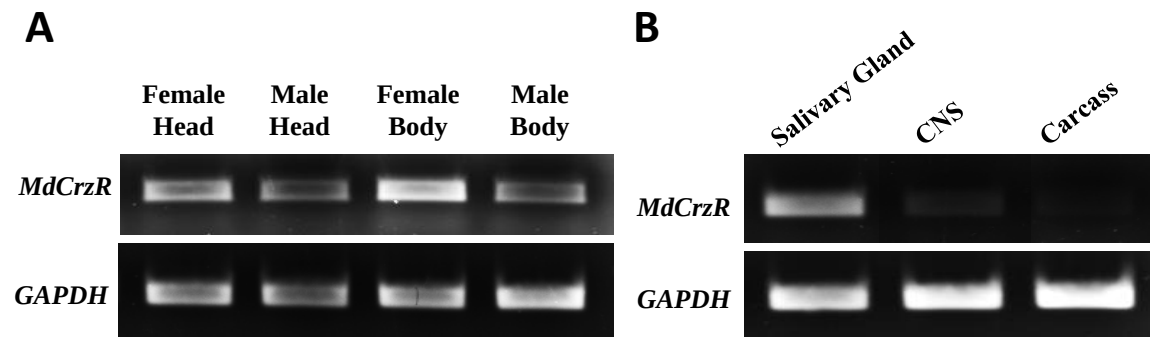


Figure 4-5. Expression of the *MdCrzR* determined by RT-PCR. As a positive control, *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) was used. (A) *MdCrzR* was amplified by RT-PCR from the three different tissues of the third instar larvae. (SG, salivary glands; CNS, central nervous system; CS, carcass). (B) RT-PCR of adult mRNA from heads or bodies (F, female; M, male).

conservation in TM domains and loop regions among distantly related CrzRs, including the DRY motif forming hydrogen bonding networks between surrounding TM helices (Park et al., 2008), which is suggested to be important for G-protein coupling selectivity (Wess, 1998). CrzRs have variable ECD and ICD sizes, which is consistent with the statistical analysis of the vertebrate class-A GPCRs that the length of the third IL varies considerably from 1 to 230 among 1,272 sequences, while the length of other loops are nearly the same (Suwa et al., 2011).

In *D. melanogaster* adults, the high-throughput analysis shows a high level of CrzR expression in the fat body, heart, and epidermis, intermediate levels in the salivary glands, and low levels in the CNS (Flybase: flybase.org). These data suggest multifunctionality (neuronal and non-neuronal roles) of the Crz particularly in adults. This notion is also supported by the neuroanatomy of the Crz neurons, which send axons into different brain areas and into the corpora cardiaca (Bagnardi et al.), a neurohemal organ (Choi et al., 2008). The former projection indicates Crz's central functions perhaps as a neuromodulator of other neurons. The latter, CC-targeting projections are likely associated with nonneuronal Crz functions in which Crz is released into the circulatory system to regulate diverse physiological events in various target tissues. In larvae, one major non-neuronal role of the Crz appears to be associated with salivary gland functions, as this tissue expresses CrzR almost exclusively outside the CNS in both *D. melanogaster* and *M. domestica*. Since the glands produce saliva to facilitate food digestion, it would be interesting to determine whether Crz signaling system regulates secretory activities of this tissue.

Chapter 5

Conclusions

In this dissertation, the functionality of Crz signaling and evolutionary aspects of *CrzR* have been discussed:

Crz and *CrzR* null alleles were generated by transposable element mobilization. Genetic and transgenic rescues were applied. Also, the structural features of the *Crz* neuron in *D. melanogaster* were presented via the FRT/FLP recombination system.

Using genetic and transgenic tools, the biological functions of the Crz signaling system in ethanol induced behavior and ethanol metabolism were investigated. Flies lacking *Crz* neurons or *CrzR* displayed significantly delayed recovery from ethanol-induced sedation. Consistent with this, *Crz* and *CrzR* were found to be required for normal ALDH activity, since the knocking-out of *Crz* neurons or *CrzR* mutation significantly reduced ALDH activity. In addition, *CrzR* shows a down-regulatory effect on ADH activity, which is initiated at transcription level through protein kinase A (PKA)-dependent pathway. Such up and down regulations of ALDH and ADH activities prevent acetaldehyde from accumulating.

To gain the evolutionary aspects of *CrzR*, *CrzR* from the House Fly, *Musca domestica* (*MdCrzR*) was cloned and characterized. *MdCrzR* is a 655-amino acid polypeptide which is characterized as

Class-A GPCR. Although the TMs and loops between the TMs are conserved in other CrzRs, the *N*-terminal extracellular domain is quite dissimilar. Tissue-specific *RT*-PCR revealed a high level of *MdCrzR* expression in the larval salivary glands and a moderate level in the CNS. In adults, the expression was broadly observed without significant gender difference, suggesting multifunctionality of the Crz signaling system.

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Appendix

Crz single neuron labeling

As described previously, there are three pairs of DL-Crz neurons in the brain lobe, and eight pairs of Crz neurons in the VNC. In order to label single Crz neurons, we took advantage of a *FLP/FRT* system, with two *FRT* sites flanking a termination codon, followed by a membrane bound GFP marker (Fig. 6-1A). A nucleus *RFP* marker, *Redstinger*, was used here for the visualization of Crz neurons. To do this, *Crz-gal4* line was crossed to *hs-flp;; UAS-frt-mCD2-stop-frt-mCD8GFP*; progenies were grown at 18°C and incubated at 37°C water bath for 1 h before pupariation. Anti-CAP was used for detecting Crz neurons. CNS from 3rd instar larvae were dissected, followed by a procedure for Immunohistochemistry. Differential fluorescent signals were captured by Olympus BX-61 microscope equipped with a CCD camera and then merged.

Both *mCD8GFP* and *Redstinger* were expressed under the control of *Crz-gal4* driver. In the presence of flippase induced by heat shock promoter, cells that undergo recombination between the two *FRT* sites eliminate the stop codon that is immediately upstream of the *mCD8GFP* coding region. Thus, *UAS-mCD8GFP* is capable of producing transcripts from the *Crz-gal4* driver. Those *GFP* positive Crz neurons become the only cells in *GFP* negative animals and hence are uniquely labeled on both neuronal soma and projections. The other Crz neurons were labeled with only *Redstinger* in the nucleus.

In the ventral ganglia, the *Redstinger* labeled Crz neuronal somata are ventrally located (Fig. 7-1B-H). The neurons send projections contralaterally to their bilaterally symmetrical cells across the midline, and then direct anteriorly or posteriorly, depending on their segmentation, to arborize in different brain regions. Two major short branches from these projections direct dorsally, parallel to the midline. After crossing the midline, the first three pairs from the posterior side of the CNS, namely abdominal 4-6 (A4-6), send their axonal projections to the posterior part of the bilateral side (Fig. 6-1B, C). The other five pairs of vCrz neurons (T2-3, A1-3) direct anteriorly, fasciculating a longitudinal tract with the other ipsilateral neurons (arrow head in Fig. 6-1D-H). The anterior end

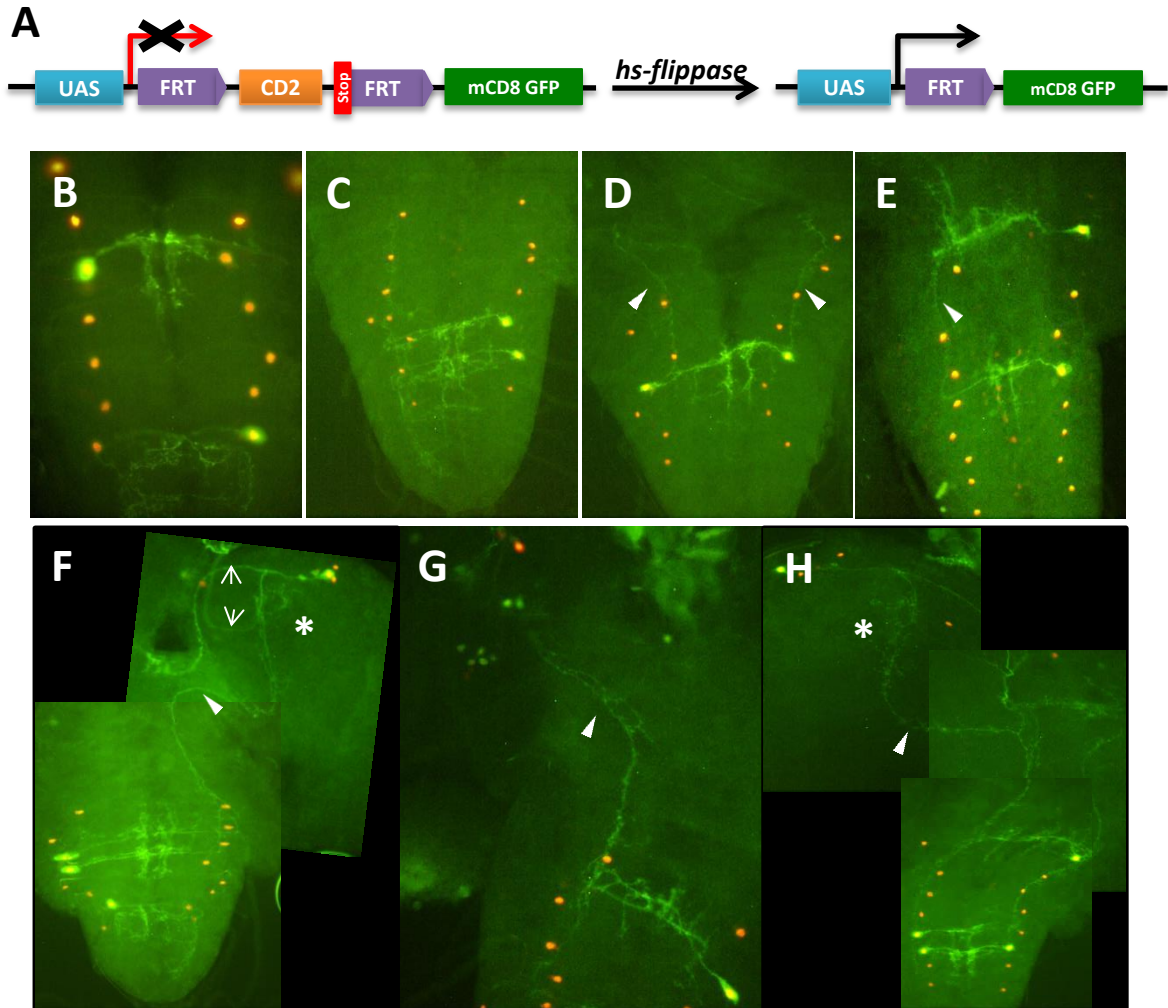


Figure 6-1. Characterization of *Crz* neurons. (A) Mosaic analysis of *Crz* neurons using FLP/FRT system. A transcriptional stop sequence is placed immediately upstream of the coding sequence of *mCD8GFP*. Deletion of the stop cassette by heat shock induced flippase expression initiates *mCD8GFP* expression. (B-H) Single *Crz* neuron labeling. *UAS-frt-stop-frt-mCD8GFP;;UAS-Redstinger* was expressed under the control of *Crz-gal4* driver. The somata of all *Crz* neurons were labeled with *Redstinger* (red). The neurons that underwent FLP/FRT recombination were also labeled with membrane bound GFP (green).

of this tract arborizes in a brain region where DL fibers bifurcate (asterisk in Fig. 6-1F, H), indicating possible interactions between DL and VNC neurons. Axon projections from the DL neurons initially extend to the medial brain region and then separate into two branches (arrows in Fig. 6-1F); one of them continues projecting medially up to the midline, then extends posteriorly to border the esophageal foramen. The other branch travels posteriorly, then makes an anterior turn, forming a loop, and finally terminates at the ring gland.

Vita

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