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The regulation of neuropeptide *Corazonin* and its functional analyses in *Drosophila melanogaster*

A Dissertation

Presented for the

Doctoral philosophy

Degree

The University of Tennessee Knoxville

Seung-Hoon Choi

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Dedication

This dissertation is dedicated to my family in Korea; they have always encouraged me with their love.

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I would like to thank all who helped me to complete my studies. I thank my advisor, Dr. Jae H. Park for providing scientific support, guidance, and his help with my dissertation. I appreciate all members of my dissertation committee: Dr. Ranjan Ganguly, Dr. Mariano Labrador, Dr. Ana Kitazono, and Dr. Jung Han Kim, for their advice, encouragement, and guidance. I am also grateful to all members in Dr. Park's laboratory, especially Dr. Gyunghee Lee, who gave me scientific insights.

Abstract

Neuropeptides regulate diverse physiological processes, including homeostatic metabolism, behavior, reproduction, and development. The neuropeptide *Corazonin* (*Crz*), was first isolated from American cockroach, *P. americana*, as a potent cardioactive substance, and has been shown to exert diverse functions in different insects. In *Drosophila*, *Crz* expression is limited to three groups of neurons; totaling only 26 neurons out of ~10,000 neurons in a third instar larval central nervous system (CNS). In adults, *Crz* is expressed in 6-8 pairs of protocerebral neurons and 2 pairs of male specific abdominal ganglion. To gain insight into such tight regulatory mechanisms of *Crz* gene transcription, *Crz* promoter activity was dissected *in vivo*. The promoter bashing experiments yielding various 5'-upstream sequences show that there are separate *cis*-acting elements that are highly conserved phylogenetically, which speaks to its functional significance in the activation of *Crz* transcription. In larval stage, a 504-bp upstream region is sufficient to activate *Crz* in all endogenous neurons. Further dissection revealed two important regions; one between -419-bp and -504-bp region for the expression in dorsal medial neuron (DM). The other located between -241-bp and -380-bp is responsible for dorsal lateral (DL) and ventral nerve cord (VNC) expression. The latter region can be subdivided into three DL-specific and two VNC-specific *cis*-acting elements. For DL-specific expression, two out of any three combination were needed; however, VNC needed two elements altogether. Interestingly, basal transcription factor binding site TATA box showed minor role for *Crz*

expression. In contrast to the larval expression, 321-bp upstream region is sufficient to activate *Crz* in all adult neurons. For the male-specific abdominal ganglion (*ms-aCrz*) expression, the *cis*-acting element was found to be in a region between -250-bp and -290-bp. Overall, the data show that transcriptional regulatory mechanisms for *Crz* expression are not uniformed among *Crz*-containing neurons, which further indicates that their neuronal functions might be different.

To identify the roles of *Crz* in *Drosophila*, several fly behaviors were tested; ethanol-related responses, olfactory sensing responses and circadian rhythmic behaviors. *Crz* cell deficient (Crz-CD) flies and *Crz receptor* knock down (CrzR-KD) flies showed significantly delayed recovery from ethanol-induced sedation compared to control flies. Such hangover phenotype was ethanol specific. This result suggests that *Drosophila Crz* involves in ethanol-related responses. Further analyses suggest that Crz-CD, CrzR-KD and *CrzR* mutation did not affect aldehyde dehydrogenase (ALDH) at transcription level, but reduced ALDH enzyme activity. *Crz* is also associated with olfactory signaling, as Crz-CD and CrzR-KD flies are unable to find odor source, such as live yeast paste. Previously, Crz neurons located in the vicinity of nerve terminals originated from circadian pacemaker *Pdf*-expressing neurons, which indicate *Crz* neurons as part of circadian circuit. However, circadian locomotor rhythmic behavior of Crz-CD, Crz over-expression and CrzR-KD flies show normal circadian rhythmic behavior.

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Abbreviations

A. cuprea: Anomala cuprea

AC: Adenylate Cyclase

ADH: Alcohol Dehydrogenase

A. I.: Attraction Index

Akh: Adipokinetic hormone

ALDH: Aldehyde Dehydrogenase

amn: *amnesiac*

APF: After Puparium Formation

bHLH: basic Helix-Loop-Helix

bZIP: basic-leucine Zipper

*C. elegan*s: *Caenorhabditis elegans*

CAP: Crz-Associate Peptide

cAMP: cyclic Adenosine Mono-phosphate

CC: Corpora Cardiaca

Clk: *Clock*

CNS: Central Nervous System

CREB, cAMP Response Element Binding

Crz: *Corazonin*

Crz-CD: Crz Cell Deficiency

CrzR: Crz Receptor

CrzR-KD: Crz Receptor Knock-Down

cyc: *cycle*

CRY: CRYPTOCHROME

CYP: Cytochrome P450

fax: failed axon connection

GABA: γ-amino-butyric acid

dbt: double-time

D. busckii: Drosophila busckii

DD: constant darkness

DL: Dorso Lateral

DLP: Dorso-Lateral Posterior

DM: Dorso Medial

DpCrz: *Drosophila pseudoobscura Crz*

DvCrz: Drosophila virilis Corazonin

EcR: Ecdyson Receptor

EMSA: Electrophoretic Mobility Shift Assay

ELAV: Embryo Lethal Abnormal Vision

EtOH: Ethanol

ETH: Ecdysis-Triggering Hormone

GFP: Green Fluorescent Protein

GPCR: G-Protein Coupled Receptor

Hid: Head Involution Defective

iGluRs: Ionotropic Glutamate Receptors

IP3: Inositol triphosphate

IR: Inverted Repeat

IRs: Ionotropic Receptors

LD: Light and Dark

LNs: Lateral Neurons

LN_d: Dorsolateral Neuron

LN_v: Ventrolateral Neuron

l-LN_v: large ventro-lateral neuron

mClock: *mouse Clock*

Mef2: Myocyte enhancer factor 2

mPer: mouse Period

M. sexta: Manduca sexta

ms-aCrz: male-specific abdominal ganglion Crz

nau: *nautilus*

NMDA: N-methyl-D-aspartate

NAD: Nicotinamide Adenine Dinucleotide

NPY: Neuropeptide Y

NPF: Neuropeptide F

NPFR: Neuropeptide F Receptor

OL: Optic Lobe medulla

OR: Olfactory Receptor

ORNs: Olfactory Receptor Neurons

Ox-Crz: Crz overexpression

PAS: PER-ARNT-SIM

PBS: Phosphate Buffered Saline

PCR: Polymerase Chain Reaction

PDE: Phosphodiesterase

PDF: Pigment-Dispersing Factor

PEP: Protein on Ecdyson Puff

per: *period*

PLC: Phospholipase C

PLD: Phospholipase D

PTEH: Preecdysis-Triggering Hormone

RNAi: RNA interference

PNs: Projection Neurons

PKA: Protein Kinase A

ROS: Reactive Oxygen Species

RT-PCR: Reverse Transcriptase-Polymerase Chain Reaction

rut: *rutabaga*

tim: timeless

sgg: shaggy

s-LN_v: small ventro-lateral neuron

vri: vrille

UTR: Untranslated Region

VNC: Ventral Nerve Cord

vCrz: Crz in the ventral nerve cord

Chapter One

Background and Significance

I. Overview of neuropeptide

Neuropeptides are small reactive peptides that are produced by specialized neurons, neurosecretory cells and regulate a wide range of physiological processes, including homeostatic metabolism, behavior, reproduction, and neuronal signal transduction pathways in animals (Li et al., 1999; Sandman et al., 1999; Nassel, 2002; Li et al., 2003). Some neuropeptides are produced by the neurons as well as endocrine cells present in peripheral tissues, such as gut. These small molecules find to their cognate cell surface receptors and activate signal transduction pathway (Grimmelikhuijzen et al., 1996).

Most neuropeptides are initially produced as precursors or prohormones and then processed by several enzymatic reactions, including cleavage by prohormone convertase, amidated, and pyroglutaminated (Strand, 1999). The mature peptides are packaged in the secretory vesicles, and then released from the nerve terminals upon stimulation.

The structures and functions of neuropeptides are maintained throughout evolution; for example, neuropeptide Y (NPY) is known to regulate food intake and energy balance. Vertebrate NPY proteins consist of 36 amino acids and show extraordinary amino acid sequence conservation from mouse to human (92%, Blomqvist et al., 1992; Brown et al., 1999; Strand, 1999). *Drosophila* neuropeptide F

(NPF), which is a mammalian NPY homolog, also has 36 amino acids residues as vertebrate NPY. This protein is structurally and functionally conserved in evolution (Brown et al., 1999; Garczynski et al., 2002; Wen et al., 2005).

Several groups have identified ~118 putative secretory proteins and around 200 GPCR genes for their receptors in the *Drosophila* genome. Although more than 100 genes are predicted to encode secretary proteins in *D. melanogester*, roles and regulations of the majority of these peptides are not well understood (Hewes and Taghert, 2001; Hauser et al., 2006; Liu et al., 2006).

II. *Corazonin*

CRZ, one of the many insect neuropeptides, was first isolated from the corpora cardiaca (CC) extracts of the American cockroach, *Periplaneta americana,* in which it stimulated the heart beat. When cultured cockroach heart tissue is treated with CRZ peptide *in vitro*, the heart beat is accelerated in a dose dependent manner (Veenstra, 1989).

Crz was also purified from several orders in insects such as Orthoptera (grasshopper and crickets, Hua et al., 2000), Lepidoptera (moths), Hymenoptera (bees and ants), and Diptera (flies and mosquitoes). The mature peptide consists of 11 amino acid residues. Three isoforms have been identified in insects; [Arg^7]-, [His^7]-, and [Thr^4, His^7]-corazonin, in which the numbers indicate the position of amino acids (Veenstra, 1991; Verleyen et al., 2006). The [Arg^7]-Crz was found in cockroach, crickets, silkworm (Hua et al., 2000), waxmoths (Hansen et al., 2001) and *Drosophila* (Veenstra, 1994), while [His^7]-Crz isoform was found in locusts (Veenstra, 1991; Tawfik et al., 1999), and

the last one was recently predicted from the *Crz* gene in honey bee, *Apis mellifera* (Verleyen et al., 2006).

Crz is a multi-functional neuropeptide in diverse insect species. For example, in several locusts species (e.g., migratory locust, *Locusta migratoria*), when CRZ was injected into albino nymph, the adult developed dark pigmentation in the cuticle, instead of cardiostimulatory effect that was observed in cockroach (Tawfik et al., 1999; Tanaka, 2001; Yerushalmi et al., 2002). In the desert locust (*Schistocerca gregaria*), CRZ injection in nymphal stage reduced the number of antennal sensilla on the 2nd, 8th, and 14th segment at the adult stage (Maeno et al., 2004; Maeno and Tanaka, 2004).

Crz also plays a role in the development and behavior in lepidopteran and dipteran insects. Injection of CRZ into *Bombyx mori* fifth instar larvae, that stop feeding and started wandering for cocoon production, reduced spinning rate during pupal formation (Tanaka et al., 2002). In tobacco hornworm, *Manduca sexta*, *Crz receptor* transcripts are detected in Inka cells, which are endocrine cells that release preecdysis-triggering hormone (PTEH) and ecdysis-triggering hormone (ETH). The expression of *CrzR* is activated by Crz just before PTEH releasing. Injection of synthetic Crz into pharate larvae also induces preecdysis and ecdysis behavior through [Ca^{2+}] and [cGMP] elevation, suggesting *Crz* involved in the initial stage of ecdysis (Kim et al., 2004). *Crz* also seems to be important for developmental arrest (diapause) when conditions are not favorable. Low temperature and short day photoperiod causes *Manduca sexta* larvae to undergo pupal diapause. Removal of putative *Crz* expressing cells by surgical ablation disrupts pupal diapause under this condition (LD=10h:14h at 22°C, Shiga et al., 2003). Similarly, in female blowfly, *Protophormia terraenovae*,

surgical removal of the pars lateralis (PL) containing *Crz* neurons did not produce ovarian diapause under short day and low temperature condition (Shiga et al., 2003). These findings suggest that *Crz* producing neurons are associated with the detection of seasonal changes.

III. *Corazonin* in *Drosophila*

Genus *Drosophila* has three subgenuses, which are estimated to have evolved about 60 million years ago (Powell and Moriyama, 1997). The subgenus *Sophophora* includes *D. melanogaster*, *D. simulans* and *D. erecta,* the second subgenus *Drosophila* has *D. virilis* and *D. borealis* and the last subgenus *Dorsilopha* has *D. busckii*. Previously, *Crz* genes were cloned and characterized in several species. The *D. melanogaster*, *D. simulans* and *D. erecta*, which are members of *Sophophora*, *Crz* genes have only one intron, whereas the *D. virilis* has two introns indicating rapid evolutionary changes of *Crz* gene structure. The first intron of *D. virilis Crz* (*DvCrz*) locates within 5' untranslated region (UTR) and the second one is in the open reading frame (Choi et al., 2005).

Prepro-Crz consists of three distinct regions: signal peptide, Crz and Crz-associated peptide (CAP). The Crz protein precursor, in *D. melanogaster*, *D. simulans* and *D. erecta* has 19 amino acids as a signal peptide and 121 amino acids as a CAP. However, the *D. virilis* has 20 amino acids as a signal peptide and 119 amino acids as a CAP. Despite such structural difference, *Crz* has a consensus proprotein processing site (GKR region, Fig. 1-1). The amino acids in CAP region of the *Sophophoran* subgenus share with more than 90%, but show only 38~ 42% similarity with the *DvCrz*

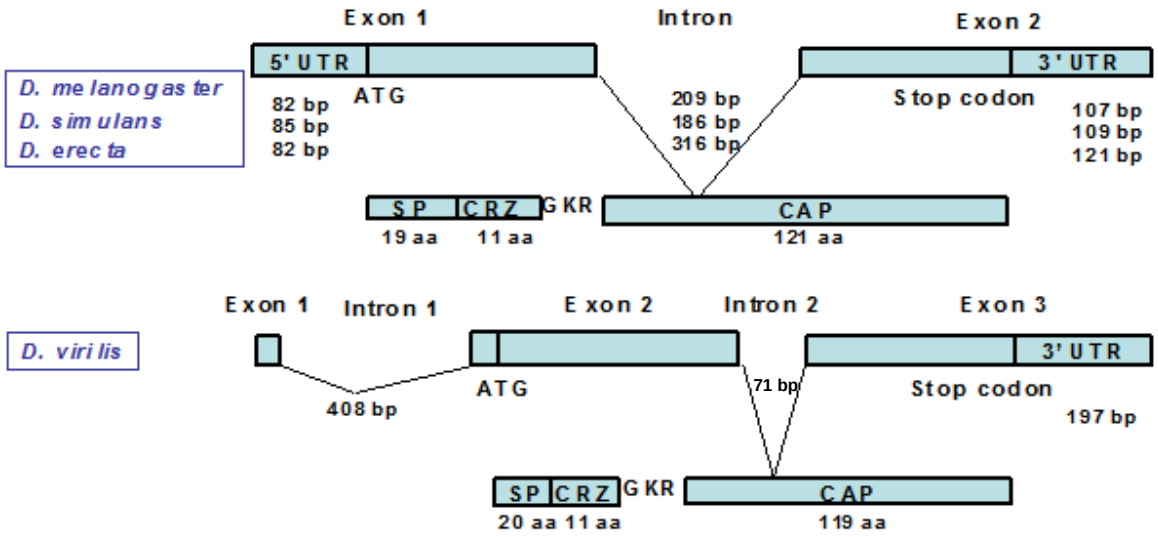

Fig. 1-1. Gene and protein structure of *Corazonin* in *Drosophila* species. *Crz* precursors of *D. melanogaster*, *D. simulans* and *D. erecta* consist of N-terminal signal peptide (19 amino acids long), Crz peptide (11 amino acids) and corazonin-associate peptide (CAP). Consensus processing site (GKR) exists in all pro-Crz peptide. *D. melanogaster, D. simulans* and *D. erecta* have one intron located in the CAP encoding region. *D. virilis* has an additional intron exists in 5' UTR region (+72-bp from transcription start site). Figure modified from *Choi et al*., 2005

(Choi et al., 2005).

IV. *Corazonin* expression pattern in larval and adult CNS

In situ hybridization of CNS from *Drosophila* species with specific RNA probe showed almost identical expression patterns (Choi et al., 2005). In the third instar larva, 24 cells showed *Crz* expression; one pair of dorsomedial (DM) neuron, three pairs of dorsolateral (DL) neurons in brain region and eight pairs of ventral nerve cord (VNC). Exceptionally, *D. virilis* showed one more pair of cell expression (Fig. 1-2C).

During metamorphosis, *Crz* expression pattern changes dramatically. The vCrz cells disappear within 6 hours after puparium formation (APF). The loss of vCrz cells is due to programmed cell death, which is initiated by ecdysone signaling involving EcR-B isoforms (Choi et al., 2006)

In the adult stage, 6 to 8 of Crz expressing neurons are located in dorso-latero-posterior (DLP) region in each hemisphere. Interestingly, old flies (more than 12 days) showed the mRNA signal in the optic lobe, but the proteins are not detectable by immunohistochemistry, raising the possibility that the mRNAs are not translated. Of interest the mRNA expression in the optic lobe is found only in *D. melanogester* (Choi et al., 2005). Recently, four adult neurons were found to express *Crz* in the abdominal ganglion, and this expression is male-specific (Lee et al., 2008). Overall, *Crz* expression is regulated by cell specific, gender-specific, and developmental stage specific factors.

Since *Crz* is likely to be associated with ethanol-related behavior, olfaction, and circadian rhythms, I will overview each of these physiological events.

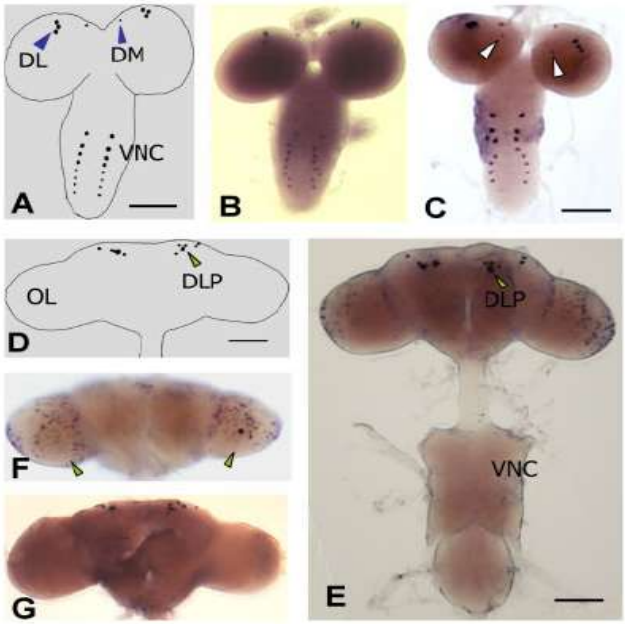

Fig 1-2. *In situ* hybridizations of *Crz* transcripts. (A) Schematic diagram of *Crz* transcript of larval CNS in *D. melanogaster*. (B) A representative image of larval CNS. One dorsomedial neuron (DM), three dorso lateral neurons (DL) in each hemi brain and eight pairs of vCrz neurons. (C) *D. virilis* larval CNS. One extra cell in medial region of brain expresses *Crz* (arrowhead). (D) Diagram of adult *Crz* expression. (E) A representative image of adult CNS in *D. melanogaster*. Male specific neurons in the abdominal ganglion are not shown in this image. (F) Widespread signals of *Crz* transcripts in the medulla optic lobe in old adult brain. (G) *D. virilis* adult brain does not show any signal in the medulla. Figure modified from *Choi et al*., 2005

V. Ethanol related behavior

Alcohol is everywhere and alcohol consumption is a prehistoric event. Drinking ethanol affects human's physiology, behavior, psychology and even economy and sociology (Bellen, 1998; Weiss and Porrino, 2002; Lovinger and Crabbe, 2005). At low concentrations, alcohol serves as wonderful beverage to cause euphoria and loss of social inhibition; but at high concentrations, alcohol induces loss of body control, dehydration, sedation, hangover and even death. Chronic alcohol consumption causes neuroadaptation in brain to lead alcohol abuse and addiction. Neuroadaptation requires repeatedly exposure and increasing amounts of alcohol (Weiss and Porrino, 2002; Heinz et al., 2003).

Ethanol is an amphiphilic molecule, therefore it passes freely cellular membrane, suggesting that ethanol affects in the brain non-selective manner. However, the main targets of alcohol are the membrane-associated molecules including N-methyl-D-aspartate (NMDA), γ-amino-butyric acid (GABA), serotonin receptors, calcium and potassium channels, and adenosine transporter (Diamond and Gordon, 1997; Dohrman et al., 1997; Gordon et al., 1997; Bellen, 1998). In addition, several G-protein coupled receptors (GPCRs), including dopamine, opioid, and adenosine receptors, are up- or down-regulated by ethanol exposure (Gonzales et al., 2004; Nestler, 2005). Alcohol abuse or addiction shares common neural substance with other addicting drugs at the molecular, cellular levels. However, alcohol addiction has distinct characteristics comparing to other addictions (Lovinger and Crabbe, 2005).

Ethanol treatment of cells and tissue culture affects GPCR mediated cyclic AMP (cAMP) pathways (Tabakoff and Hoffman, 1996). In acute exposure to ethanol, cAMP

synthesis is accelerated, but in chronic exposure, cAMP production is decreased. This reduction in cAMP mediated signal transduction may be due to desensitization of the $G_{\alpha s}$ or activation of the $G_{\alpha i}$ subunit. Elevation of cAMP level activates protein kinase A (PKA) and leads to cAMP response element binding protein (CREB) activation to produce target genes (Diamond and Gordon, 1997; Dohrman et al., 1997; Nestler and Aghajanian, 1997). Expression of PKA inhibitor transgene in the mice fore brain or mice lacking one copy of the gene encoding $G_{\alpha s}$ displays hypersensitivity to ethanol (Wand et al., 2001). Despite these studies, target neurons or regions of alcohol in the brain are still not known.

Ethanol is metabolized into a non-toxic chemical, acetic acid, via acetaldehyde by cytosolic enzyme alcohol dehydrogenase (ADH, E.C. 1.1.1.1., Grover et al., 1998; Fry et al., 2004; Fry and Saweikis, 2006) and cytosolic (type I) or mitochondrial (type II) aldehyde dehydrogenase (ALDH, E. C. 1.2.1.3., Heinstra et al., 1989; Zakhari, 2006).

Many *Drosophila* species breed in fermenting fruit, where ethanol concentrations can reach several percent or may be even higher (McKenzie and McKechnie, 1978; Gibson and Oakeshott, 1981). Thus flies have evolved to tolerate high ethanol content. The ethanol resistance is one of the important characters as a selective marker for *Drosophila* interspecific comparison (Mercot, 1994). *D. melanogaster* is one of the most resistant species to ethanol, and are actually attracted to ethanol (David and Van Herrewege, 1971; David and Bocquet, 1975; Parsons and King, 1977). The ADH, which catalyses the oxidation of ethanol to acetaldehyde using nicotinamide adenine dinucleotide (NAD^+) as a cofactor, is the essential for ethanol degradation and resistance. Several ADH isoenzymes are identified: for example, human has five ADHs

(Thomasson et al., 1995; Agarwal, 2001). *Drosophila* has only one ADH (Freriksen et al., 1991; Heinstra and Geer, 1991; Pecsenye and Saura, 1998), and *Adh* null flies are extremely sensitive to ethanol (David et al., 1976; Bijlsma-Meeles and Bijlsma, 1988; Oudman et al., 1991). Thus ADH activity is correlated with ethanol resistance among *Drosophila* species (Mercot, 1994). Within *D. melanogaster*, strains with the more enzymatically active *Fast* electoromorph show higher ethanol resistance than less active *Slow* electoromorph (reviewed in Heinstra, 1993).

Another important enzyme in ethanol metabolism is ALDH, which catalyses the irreversible NAD^+-dependent oxidation of acetaldehyde to acetic acid. ALDH is considered the main enzyme responsible for the oxidation of acetaldehyde in mammals (Tank and Weiner, 1979; Weiner, 1982, 1987). Several ALDH isoenzymes are isolated, but only cytosolic ALDH1 and mitochondrial ALDH2 have activities. Aldehydes are converted to acetate mainly by ALDH2, which belongs to the phase I drug-metabolizing enzyme, in the mitochondria (Mizoi et al., 1994; Isse et al., 2005; Zakhari, 2006). In humans, an inherited deficiency of ALDH2 causes a syndrome known as acute alcohol sensitivity, which is more prevalent in the Eastern Asian population (Impraim et al., 1982; Yoshida et al., 1984; Peng et al., 1999). After ingesting small amounts of ethanol, these people experience a variety of unpleasant symptom caused by the accumulation of acetaldehyde, which is considerably more toxic than ethanol.

While *Drosophila* has an active ALDH (Lietaert et al., 1985; Garcin et al., 1986; Heinstra et al., 1987, 1989; Anderson and Barnett, 1991; Anderson et al., 1991; Leal and Barbancho, 1993), some research groups suggested that ALDH is less important for ethanol metabolism in *Drosophila* than mammals (Geer et al., 1985; Heinstra et al.,

1989), as they claimed that *Drosophila* ADH is capable of oxidizing acetaldehyde to acetate by itself (Brooks et al., 1985; Eisses et al., 1985; Eisses et al., 1985; Geer et al., 1985; Heinstra et al., 1986, 1987, 1989). Unlike mammalian ADH, however, *Drosophila* ADH has quite different structure and have weak ability to oxidize aldehyde, suggesting that *Drosophila* ADH has different evolutionary pathway (Hinson and Neal, 1972).

During ethanol degradation, reactive oxygen species (ROS) are generated as byproducts. To protect cell and tissue damage from ROS attack, several enzymes are involved in ethanol metabolism: for examples, xenobiotic enzyme Cytochorome P450 (CYP), catalase, and phospholipase D (PLD, Reginato et al., 1998; Zakhari, 2006).

Like mammals, *D. melanogester* shows quite similar responses to ethanol with respect to the behavioral patterns (Bellen, 1998; Heberlein, 2000; Scholz et al., 2000; Guarnieri and Heberlein, 2003). At low doses, flies increase locomotion, but at high doses, their movement is gradually decreased and finally intoxicated or sedated. In addition, flies acquired tolerance after a single dose treatment according to dose dependent manner, which is arbitrated by adaptation of brain activities (Scholz et al., 2000).

At least two neuropeptides are involved in ethanol-associated physiological events in flies. Neuropeptide F (NPF), a mammalian neuropeptide Y (NPY) homolog, is involved in ethanol resistance. Deficient in NPF/ NPF receptor (NPFR) showed decreasing alcohol sensitivity; conversely, overexpression of NPF/NPFR decreased ethanol resistance (Wen et al., 2005). Another fly neuropeptide *amnesiac* (*amn*) also participates in ethanol sensitivity. The *amn* mutant shows malfunction of memory (Quinn et al., 1979) and hypersensitivity to ethanol (Moore et al., 1998). The product of

amn gene, AMN, stimulates cAMP synthesis and activates protein kinase A (PKA) signal transduction pathway (see Fig. 4-1; Bellen, 1998; Moore et al., 1998). Consistently, adenylate cyclase (AC) loss-of-function mutation, *rutabaga* (*rut*^{769}) and a loss-of-function mutation of cAMP-dependent protein kinase A catalytic subunit (PKA-C), *DCO*, induced hyperactivity and premature sedation (Moore et al., 1998). In contrast, fly cAMP phosphodiesterase (*dunce*) mutant increases levels of cAMP, but it shows similar sensitivity to wild-type control fly (Davis and Laroche, 1996).

Recently, production of *Drosophila* Homer protein, a homolog of mammalian Homer 1, is required for normal ethanol sensitivity and tolerance, as the *Homer* mutant flies increase the ethanol sensitivity and fail to develop tolerance after ethanol exposure (Urizar et al., 2007). Like mammals, repeated ethanol exposure not only induces tolerance to the sedation, but also increases male to male courtship (Lee et al., 2008). These similar responses to ethanol between mammals and flies strongly suggested that ethanol affects fly and mammalian nervous system in a similar manner.

VI. Olfactory response in animal

Chemical perception provides animals with information about the locations of food, mating partners, and oviposition sites. The olfactory system of insect and mammals exhibits many similarities, suggesting that fundamental mechanisms for olfactory signaling may be shared. In *Drosophila*, response to attractive and repulsive compounds has been studied (Alcorta and Rubio, 1988, 1989; Alcorta, 1991). However, the mechanism of olfactory perception is poorly understood.

In adult fly, each olfactory sensillum is loaded with one to four olfactory receptor neurons (ORNs). The pores of cuticles on the sensillum permit passage of chemicals to access specific odorant receptors (ORs; Stocker, 1994; Stortkuhl et al., 1994; Kim and Smith, 2001; Warr et al., 2001; Gendre et al., 2004; Dahanukar et al., 2005). The initial steps in odor detection involve the binding of a chemical to OR proteins displayed on ciliated dendrites of specialized ORNs. The OR genes are expressed in subpopulations of ORNs (Clyne et al., 1997, 1999a, 1999b; Larsson et al., 2004), and are members of a rapidly diverging superfamily of insect chemosensory genes that encode seven transmembrane domain receptors with no homology to nematode or vertebrate ORs (Hill et al., 2002a, 2002b). The odor response profile of each ORN is governed by the selective expression of one or more ORs out of 60 OR genes.

In *Drosophila*, about 1300 ORNs distribute in the antenna and maxillary palp on each side of the head and project axons to the antennal lobe, where they terminate in ~50 morphologically discrete and synapse-dense processing modules known as glomeruli (Larsson et al., 2004; Neuhaus et al., 2005). The projection patterns of the ORNs are stereotyped between animals; ORNs that express the same OR gene project their axons to the same glomerular target. The olfactory signal from peripheral sense organ transfers to olfactory center by two types of neurons; the local interneurons (LNs) and the projection neurons (PNs). The soma of about 150 PNs and 100 LNs exists in each antennal lobe (AL). The LNs, primarily GABAnergic inhibitory neurons, are axonless, and have broad, multiglomerular ramifications within the AL. The PNs pass the olfactory information from AL to olfactory center, such as mushroom bodies for learning and memory or lateral horn of the protocereberum (Wilson and Laurent, 2005).

A unique feature of the circuitry within the insect AL is the presence of reciprocal dendrodendritic connections between the PNs and the LNs. The presence of such unique junctions with both transmissive and receptive synapses provides anatomical evidence that each glomerulus makes computations that may underlie odor perception, discrimination, and learning, rather than being a simple transit station for the throughput of olfactory information. Individual PNs generally extend dendrites into a single antennal lobe glomerulus and then convey the processed olfactory information to the higher order olfactory neurons (Davis, 2004).

VII. Olfactory receptors (ORs)

As previously described, the volatile chemicals are detected by ORs. In rodents, the discovery of multigene family of ORs was made by Buck and Axel (1991). In mammals, OR genes encode 500-1,000 (Buck and Axel, 1991). These receptors are expressed mainly in the nasal olfactory epithelium of mammals (Buck and Axel, 1991; Selbie et al., 1992; Rouquier et al., 1998), birds (Deutsch and Nefdt, 1992; Nef et al., 1992; White et al., 1992; Holzenberger et al., 1996; Leibovici et al., 1996), reptiles (White et al., 1992), amphibians (Freitag et al., 1995) and fish (Ngai et al., 1993; Ngai et al., 1993; Riddle et al., 1993). In nematode, *C. elegan*s, each of 32 chemosensory neurons expresses a large number of different ORs. In vertebrate, ORs subdivided into two groups, Class-I (fish like) and Class-II (tetrapod-like) ORs. Class-I ORs are specified for binding water soluble odorants, but Class-II ORs recognize air-born odorants (Glusman et al., 2000; Glusman et al., 2001). The class-I ORs are identified in rat and human (Feingold et al., 1999). In human, ca 1,000 OR genes are predicted by

Human Genome Project. Interestingly, approximately 60% of human OR genes are likely to be puesdogenes (Venter et al., 2001; Zozulya et al., 2001). This is contrasted with less than 5% of puesdogenes in rodents. All ORs are GPCRs, and share amino acid sequences and structures (Vassar et al., 1993; Menco and Jackson, 1997; Mombaerts, 1999; Ronnett and Moon, 2002).

Insect ORs have some different characteristics from vertebrates' ORs. First, they are highly diverse, sharing less than 20% amino acid similarity among insects ORs (Robertson et al., 2003; Hallem and Carlson, 2006; Hallem et al., 2006). One member of the ORs, *Or83b*, is strikingly different from other ORs. Unlike the conventional ORs, *Or83b* has clear homologs in other insect species that share nearly 70% amino acid identity (Hill et al., 2002; Pitts et al., 2004). Of interest, *Or83b* is coexpressed with conventional ORs in a large proportion of OSNs (Pitts et al., 2004). Second, insect ORs belong to a new category of GPCR subfamily, as the membrane topology of insect ORs is distinct with conventional GPCRs. The N terminus of insect ORs locates cytosolic side (Benton, 2006; Benton et al., 2006; Wistrand et al., 2006). Third, insect ORs consists of a heterodimeric complex with *OR83b* (Hill et al., 2002; Larsson et al., 2004a, 2004b; Pitts et al., 2004; Neuhaus et al., 2005; Benton, 2006).

Insect ORs act as an odor-gated ion channel, suggesting that they are mechanistically distinct from vertebrate ORs, which activate cAMP production and induce cyclic nucleotide-gated ion channel opening (Sato et al., 2008; Wicher et al., 2008). Surprisingly, researchers found new family of ORs. These receptors shared ligand binding domain of Ionotropic glutamate receptors (iGluRs), which are important for glutamate binding and opening ion channel (Mayer and Armstrong, 2004). From

microarray screening, six iGluR-related genes, which named ionotropic receptors (IRs), are expressed in the antenna in *Drosophila*. BLAST search using the six IRs as queries, *Drosophila* genome is revealed to have 61 predicted IRs. These IRs are expressed in coleoconic sensilla with OR35a and OR83b; however, IRs are not expressed in basiconic and trichoid sensilla. Misexpression of IRs in different ORNs is sufficient to induce ectopic olfactory sensitivity. For example, the ac3 sensilla do not show any response to phenylacetoaldehyde. When IR84a, which is expressed in ac4 sensilla and activated by phenylacetoaldehyde, is expressed in ac3 sensilla by an *OR35a-gal4* driver, the ac3 sensilla become responsive to phenylacetodadehyde (Benton et al., 2009; Spletter and Luo, 2009).

Upon odorant binding in mouse, the receptors change their structures and activate an olfactory specific G-protein (Gαolf), which turns on adenylyl cyclase type III (ACIII). The increase of cAMP induces opening of the calcium channel, tetrameric cyclic nucleotide gated (CNG) channel (Firestein, 2001). Calcium influx via CNG channels conducts chloride channel opening and results in the depolarization of membrane potentials (Restrepo et al., 1996; Reisert et al., 2005; Pifferi et al., 2006). However, phopholipase C (PLC)- inositol triphosphate (IP3) pathway in ORs is not well understood yet (Elsaesser et al., 2005; Imai and Sakano, 2008)

VIII. Biological clock

Every life on earth is affected and adapted by forced environmental cues, such as daily changes in lighting, temperature, humidity and other physical parameters. Instead almost all reaction reflects passively to the change of parameters, organisms

have endogenous clock, permitting them to prepare change of environmental daily conditions. The clock synchronizes biological rhythms, with certain phases in the 24-hour cycles, such as metabolic and physiological fluctuations. In the absence of environmental rhythmic fluctuations, the endogenous clocks are still able to maintain ca. 24-hour rhythmicity, which is referred to as circadian rhythms (Ouyang et al., 1998).

The first genetic approach of circadian clock was performed for plants by Bunning, 1954; Bunning and Moser, 1968). The study achieved the individual selections and breedings based on the length of the timing to respect external light and dark (LD) cycle. Such traits inherited to the next generations, indicating genetic components in circadian timekeeping. Genetic screenings identified 'clock genes', in the fruit fly, *D. melanogaster*, (Konopka and Benzer, 1971; Bruce, 1972; Feldman and Bruce, 1972), algae, *Chlamydomonas reinharditill*, (Bruce, 1972; Feldman and Bruce, 1972), fungi, *Neurospora crassa*, (Feldman and Hoyle, 1973), and mouse, *Mus musculus*, (Vitaterna et al., 1994).

During the past few decades, discovery of circadian clock genes and mechanisms have been greatly expanded. In *Drosophila*, clock-related genes are revealed to be main components in central circadian rhythms; *period* (*per*), *timeless* (*tim*), *Clock* (*Clk*), *cycle* (*cyc*), *vrille* (*vri*), *double-time* (*dbt*), *cryptochrome*, *casein kinase 1* and *shaggy* (*sgg*). The proteins CLK and CYC are belonged to bHLH-PAS transcription factors (bHLH stands for basic helix-loop-helix domain and PAS stands for PER-ARNT-SIM, which are PAS protein family; Crews and Fan, 1999) and form a heterodimer to bind to specific recognition sequences within the promoters of the *per*, *tim*, and *vri* genes to activate their transcription. The core mechanism of circadian clock

is molecular feedback loop in which transcriptional activation of clock genes is repressed by other clock proteins. During early morning, CRYPTOCHROME (CRY) proteins are activated by light and inhibit accumulation of TIM-PER heterodimer proteins via binding to TIM and leads to degradation. Late morning or early evening, *per* and *tim* transcripts reach the highest level as CLK-CYC activates both genes (Marrus et al., 1996; Rosbash et al., 1996; So and Rosbash, 1997). Subsequently, TIM begins to be accumulated in the cytosol. This cytoplasmic TIM binds to PER and protects it from degradation. Phosphorylation of PER accelerates its degradation by DBT kinase (Price et al., 1998). The stabilized PER-TIM heterodimer and TIM phosphorylation by another kinase SGG promotes nuclear transport and inhibits *per* and *tim* transcript production via binding to CLK-CYC heterodimer, which induces the loss of the DNA-binding ability (Martinek and Young, 2000; Lee, 2001; Martinek et al., 2001). Shortly after entry to nucleus, PER represses the CLK-CYC heterodimer until its degradation is occurred via PER phosphorylation by DBT kinase (Rothenfluh et al., 2000; Rothenfluh et al., 2000; Rothenfluh et al., 2000). This degradation allows new round of *per* and *tim* transcription synthesis in the late morning (See Fig. 6-1).

The feedback loop of oscillation is strengthened by another component VRI, which rhythmically represses the transcription of the *Clk* gene (Glossop et al., 1999; Hao et al., 1999). VRI is a member of basic-Zipper (bZip) transcription factors, and regulated by CLK-CYC heterodimer in a manner similar to TIM and PER (See Fig. 6-2). That is, *vri* gene is activated by CLK-CYC and repressed by PER-TIM (Blau and Young, 1999). Therefore, the highest levels of VRI protein reach at the night and lowest at the end of the day.

IV. Comparison of the circadian system between *Drosophila* and mammals

Circadian clock is highly conserved throughout the evolutions. Homozygous *Clk* mutant mice showed lengthened (28 h) free-running behavioral periods and eventually arrhythmic. Another positive regulator of feedback loop is BMAL1 (MOP3), a mammalian ortholog of fly CYC. As in flies, CLK-BMAL1 dimer is important to activate *mPer1* and *mCry* transcriptions. Although the basic mechanisms are conserved, many aspects are different between *Drosophila* and mammals. First, Clk expression is constitutive, while *Bmal1* is expressed rhythmically at the mRNA and protein levels at least in the SCN (Tamaru et al., 2000; Lee, 2001; Yagita et al., 2001; Vitaterna et al., 2006). It means that BMAL1 is a limiting factor of CLK-BMAL1 heterodimer formation and phase shifting for *mPer1* and *mCry* transcription (Lee et al., 2001). Other differences from flies are that there are 3 PERs and 2 CRYs in mammals, and PER, instead of TIM, binds to CRY as a partner.

The function of *mPer1* was revealed by targeted gene knock out (Bae et al., 2001; Cermakian et al., 2001). The mutant mice showed arrhythmic after 10-14 days in DD (Bae et al., 2001). Interestingly, *mPer1* mutant does not affect rhythmic oscillation of *mPer1*, *mPer2*, *mCry1*, and *Bmal1* transcription, which suggesting *mPer1* is important for posttranscriptional regulation rather than transcriptional feedback (Bae et al., 2001; Cermakian et al., 2001; Zheng et al., 2001).

Contrary to *mPer1*, *mPer2* of targeted knock out mice produced more severe behavioral defects; the mutant animals showed arrhythmic behavior in shorter time in constant darkness (Bae et al., 2001; Zheng et al., 2001). In addition, amplitudes of rhythmic expression of *mPer1*, *mPer2*. *mCry1*, and *Bmal1* mRNA are reduced, in the

mutant. These results indicate that *mPer2* is a main transcription regulator of mammalian clock (Zheng et al., 1999; Shearman et al., 2000a, 2000b; Zheng et al., 2001).

X. Output of Circadian Clock Information

It is not well understood how central clock functions regulate to downstream tissues (output pathway). Nueroendocrine system, which is under controlled by clock genes, involves rhythmic locomotor behavior (Park et al., 2000; Park, 2002). Insect neuropeptide pigment-dispersing factor (PDF) plays an important role in the output pathway. The *pdf* null mutant (pdf^0) flies show normal rhythmic behavior under 12-h light: 12-h dark (12:12 LD) cycle. However, the mutant flies loose their rhythmicity gradually and become arrhythmic after few days in DD (Renn et al., 1999). Moreover, *Pdf* is positively controlled by *clock* genes. CLK: CYC heterodimer activates pdf transcription; however, such activation is likely to be indirect, as *pdf* promoter does not contain E-box (Park et al., 2000). It is unknown how Pdf signal is transmitted and Pdf downstream targets. Of interest, *Crz* producing neurons are in close vicinity of the nerve terminals originated from Pdf-sLNV pacemaker neurons (Choi et al., 2005). This finding implies that Crz neurons are downstream of Pdf neurons, forming a circadian neuronal circuit.

In my dissertation, I will focus on two main areas. One is transcriptional regulatory mechanisms of neuropeptide *Crz* using genomic and biochemical approaches (Chapter 2 and 3). The other is functional analysis of *Crz* in *Drosophila*;

ethanol related responses, odor perceptions and circadian rhythmic behavior (Chapter 4-6).

Chapter Two

Spatial and Developmental Regulation of Corazonin Neuropeptide Expression

*This chapter is modified from Choi et al., 2008 in the Journal of comparative neurology.

SEUNG-HOON CHOI, GYUNGHEE LEE, PAMELA MONAHAN, AND JAE H. PARK (2008), Spatial Regulation of *Corazonin* Neuropeptide Expression Requires Multiple *cis*-Acting Elements in *Drosophila melanogaster* 507:1184–1195

I. ABSTRACT

In the larval CNS, *Crz* expression is limited to three groups of neurons: three pairs of DL neurons, a pair of DM neurons, and eight pairs of vCrz neurons. To gain insight into tight regulatory mechanisms of *Crz* gene transcription, *Crz* promoter activity was dissected in vivo. Promoter bashing experiments yielding various 5'-upstream sequences show that there are separate *cis*-acting elements that are highly conserved phylogenetically. In larval stage, 504-bp upstream region is sufficient to activate *Crz* in all endogenous neurons. Further dissection reveals two important regions; one between -419-bp and -504-bp for the expression in DM. The other located between -241-bp and -380-bp is responsible for DL and vCrz expression. The latter region can be subdivided into three DL specific and two vCrz specific *cis*-acting elements. For DL specific expression, any combinations of two out of the three are required; however, vCrz needed two elements altogether. Interestingly, basal transcription factor binding

site, TATA box, plays a minor for *Crz* expression. In contrast to the larval expression, 321-bp upstream region is sufficient to activate all adult *Crz* neuronal expression. For the male-specific abdominal ganglion (ms-aCrz), the *cis*-acting element was found to be in a region between -250-bp and -290-bp. Overall, the data show that transcriptional regulatory mechanisms for *Crz* expression are not uniform among *Crz*-containing neurons, which further indicates that their diverse neuronal functions.

II. INTRODUCTION

Most invertebrate neuropeptide-encoding genes are expressed in a countable number of neurons within the central nervous system (CNS) (reviewed by Nassel, 2002). A neuropeptide Corazonin-encoding gene (*Crz*) expression is restricted to three distinct groups of neurons, totaling 24 neuronal cells (out of 10,000) in the late embryonic and larval CNS in *D. melanogaster* (Choi et al., 2005; Lee et al., 2008). Such precise transcriptional regulation is essential for the normal function of a given peptidergic nervous system, because the system is elaborately organized to perform highly specialized functions. As such, ectopic production of the neuropeptide is likely to perturb overall functions of the CNS, which might reduce the animal's optimal fitness. For instance, fly neuropeptide F (NPF), a mammalian neuropeptide Y (NPY) homolog, involves ethanol-related behavior. Flies that an deficient in NPF/ NPF receptor (NPFR) showed more resistance to ethanol-induced intoxication, but overexpression of NPF/NPFR flies increase sensitivity to ethanol-induced sedation (Wen et al., 2005).

Transcriptional regulation is generally accomplished via unique interactions between sequence-specific DNA binding factors (i.e., *trans*-acting factors) and their

cognate *cis*-regulatory elements in the target gene promoter (Biggin and Tjian, 2001; Kadonaga, 2004). Although the *Drosophila* genome is predicted to contain ca. 100 neuropeptide genes, molecular components controlling transcription of these peptide genes are largely undefined. Despite this, several studies have identified responsible *trans*-acting regulators that either directly or indirectly modulate expression of several neuropeptide genes (Benveniste et al., 1998; Park et al., 2000; Gauthier and Hewes, 2006; Lee et al., 2006). Of interest, these studies have shown that the transcriptional regulatory mechanisms are not uniform among neurons that express the same neuropeptide. For instance, of 44 neurons expressing the *FMRFamide* gene in the larval CNS, only six Tv neuroendocrine cells are directly controlled by Apterous LIM homeodomain transcription factor (Benveniste et al., 1998). Further studies suggested that other intrinsic *trans*-acting factors, *Squeeze* and *Dimmed*, along with retrograde BMP signals from the target (dorsal neurohemal organ) are required for the specification of FMRFergic Tv neurons during embryonic CNS development (Allan et al., 2003, 2005). Likewise, two central circadian clock regulators, *Clock* (*Clk*) and *Cycle* (*Cyc*), are involved in the transcriptional activation of the *Pigment-dispersing factor* (*Pdf*) gene in four small ventrolateral neurons, but not in other *Pdf*-neurons (Blau and Young, 1999; Park et al., 2000; Park, 2002). Because a consensus binding sequence for the *Clk* and *Cyc* was not found within the *Pdf* regulatory sequence, it has been proposed that these two *trans*-acting factors activate *Pdf* indirectly (Park et al., 2000). Expression of the *neuropeptide F* (*npf*) requires sex-determination factor, *Fruitless*, as well as the *clock* factors (*Clk* and *Cyc*) in a subset of *npf*-neurons (Lee et al., 2006). In another report, a basic helix-loop-helix transcription factor, Dimmed, was implicated as a transcriptional

regulator for the expression of the neuropeptide dromyosuppressin in a cell type-specific manner (Gauthier and Hewes, 2006).

The aforementioned studies emphasize unique transcriptional machinery operates to specify the peptidergic phenotype of a subset of neurons, and further suggest functional diversity of the neurons that produce the same neuropeptide as a signaling molecule. One such example is the neurons that express *npf* (Brown et al., 1999; Stanek et al., 2002). In the larval CNS, *npf* is exclusively expressed in three pairs of neurons, two of which are located in the protocerebrum and the remaining one in the subesophageal ganglion (Brown et al., 1999; Shen and Cai, 2001; Lee et al., 2006). Interestingly, protocerebral *npf* expression is developmentally controlled (Wu et al., 2003), while the expression in the subesophageal ganglionic neurons is sensitive to high sugar content in the food (Shen and Cai, 2001). The cell type-specific responses of *npf* expression to different cues are likely to be mediated by differential transcriptional machinery that senses and transduces different types of external stimuli.

Expression of *Crz* mRNA is restricted to three distinct groups of neurons in the larval CNS; three pairs of dorso-lateral (DL) neurons and one pair of dorso-medial (DM) neurons in the protocerebrum, and eight bilaterally positioned vCrz neurons in the ventral nerve cord (Choi et al., 2005). Like other peptidergic nervous systems mentioned above, distinct neuronal architectures constituted by each *Crz* neuronal type imply that each group performs unique functions in response to different physiological inputs, which may modulate release of the *Crz* peptide from nerve terminals. As shown for other regulatory peptides, such signals can also be conveyed to the genome, thus controlling *Crz* expression transcriptionally (e.g.,Weisinger, 1995; Wu et al., 2003;

Yamamori et al., 2004; Higuchi et al., 2005). The latter hypothesis can be supported by the identification of group-specific components of transcriptional regulation. Thus, a central question we wish to answer here is whether separate *cis*-regulatory sequences exist for group-specific *Crz* expression.

We have previously shown 1.2-kb sequence upstream of the *Crz* gene contains *cis*-elements necessary for *Crz* expression in all groups of neurons in the larval CNS (Choi et al., 2006). During metamorphosis, the spatial *Crz* expression pattern changes dramatically, as vCRZ expression disappears in prepupal stage. In the adult stage, 6 to 8 of Crz neurons, which are located in dorso-latero-posterior (DLP) region, are detected in each hemisphere and two pairs of male specific neurons (ms-aCrz) in the abdominal ganglion (Choi et al., 2005; Lee et al., 2008). Here, we dissected this region to identify *cis*-elements involved in neuronal group-specific *Crz* expression. The results revealed two separate regulatory regions, one for the DM group and the other for both DL and vCrz groups. The latter region consists of three interdependent regulatory sites for DL- and two for vCrz-specific expression. We also performed further analysis for adult regulatory region. In contrast to larval *Crz* regulation, a region between 249-bp and 321-bp is sufficient for adult *Crz* expression. We also identified a separate ms-aCrz *cis*-acting element, which located between 249-bp and 290-bp region.

III. MATERIALS AND METHODS

Fly strains

Flies were raised on a standard cornmeal-yeast-agar medium at room temperature. *Yellow white* (*y w*) or *white*^{1118} (*w*^{111}*8*) strains were used as a host strain

for germ line transformation and normal control. Transgenic *UAS-lacZ* and *UAS-mCD8GFP* reporter lines were used for the detection of *Crz* promoter activities (Choi et al., 2005), and *UAS-reaper* for targeted ablation of neurons (e.g., Lee and Park, 2004; Lee et al., 2006). *D. simulans* and *D. virilis flies* were obtained from Tucson stock center. *Nautilus* (*nau*)-null mutants were collected as a trans-heterozygote (nau^{armGFP}/ nau^{188}, Wei et al., 2007). Trans-allelic hypomorph *Mef2* mutant ($Mef2^{30-5}$/ $Mef2^{44-5}$) were obtained from a cross between $Mef2^{30-5}$/ *CyO, GFP* and $Mef2^{44-5}$/ *CyO, GFP*. The progeny were raised at 18℃ (permissive temperature, Casso et al., 2000; Baker et al., 2005). At the third instar larval stage, GFP-negative trans-allelic *Mef2* mutants were collected under fluorescence dissecting stereo microscope (LEICA MZ16FA) and then performed Crz-immunohistochemistry.

Crz **promoter constructs**

Using previously defined 1.2 kb-*Crz* upstream fragment as a template (Choi et al., 2006), various *Crz* upstream constructs were generated by PCR. PCR primers are listed in the Table 2-1, and conditions and parameters are summarized in Table 2-2 and 2-3. The PCR products were cloned into the *pGEM-T* easy vector (Promega), from which *Bgl* II/ *Eco*RI fragments were subcloned into the *pPTGAL P*-element fly transformation vector (Sharma et al., 2002) to generate various *Crz* promoter-*gal4* constructs. The constructs were confirmed by sequencing reaction using dideoxy Dye Terminator Kit (ABI Prizm).

Constructs containing internal deletions (Fig. 2-1) were generated by

Table 2-1. PCR primers used for various *Crz* promoter constructs.

primers	sequence (5' to 3')
802	GGAGATCTGAGAAGAGCGTCGTGGAAAT
504	GGAGATCTGTTGTAACTAGTCCCCATATA
419	GGAGATCTCCAAGTCTAATTTGGACAT
380	CCAGATCTGGAAGAAAATTACTTTTTCG
321	GGAGATCTTGCCACCCACTTTCGGATT
287	GGAGATCTGGTAGTTTTCCAATCA
249	GGAGATCTAGCCAGCTGTCGTTGGTACA
241R with *Eco*RI	CG**GAATTC**AGCTGGTGCTCAAACTGAATGC
Δ(249 to 311)F	TTGCCTTTTGCCACCCAAGCC—AGCTGTCGTTGGT
Δ(249 to 311)R	antiparallel to Δ(249 to 311)F
Δ(241 to 279)F	CCGGGAAAAGTGGTAGTT—TCGTTGGTACAAGGGGC
Δ(241 to 290)R	antiparallel to Δ(241 to 279)F
Δ(241 to 290)F	GGATTTTGCCCGGGAA—TCGTTGGTACAAGGGGC
Δ(241 to 290)R	antiparallel to Δ(241 to 290)F
Δ(280 to 321)F	GCATTTTATTTTAAGCCTTTTGCCTTTTGCCTT—TTTCCAATCAGCAA ATGC
Δ(280 to 321)R	antiparallel to Δ(280 to 321)F
Δ(TATA box)F	CTTAATGGCTATCTAACAACGA—AGCCCGTCGAGGAGCATCAATAG
Δ(TATA box)R	antiparallel to Δ(TATA box)F
EMSA-for*	same as 380
EMSA-rev*	CACCGCCCCTTGTACCAACGACAGC

Numbers indicate nucleotide positions from the transcription start site (see also Fig. 2-1)

The primers with 'Δ' heading were used for PCR-mediated site-directed mutagenesis to delete sequences indicated by the numbers in the parentheses.

Underlined are *Bgl* II site for cloning purpose.

Bold characters are *Eco*RI site for cloning purpose.

*Primers used for making probes for EMSA (EMSA-r primer is biotinylated).

oligonucleotide-mediated mutagenesis following the methods described for either Quick Change Site-Directed Mutagensis Kit (Stratagene) or megaprimer-mediated PCR (Ke and Madison, 1997). The PCR products were digested with *Bgl* II/ *Eco*RI, and then subcloned into *pPTGAL* vector. Individual recombinant vectors were introduced into *y w* or w^{1118} host via conventional germ-line transformation (Rubin and Spradling, 1982; Spradling and Rubin, 1982). At least three independent lines bearing each construct were tested.

Identification of 5' flanking sequences of *D. simulans, D. pseudoobscura*

To obtain *D. simulans Crz* 5' regulatory sequence, PCR was performed using a *DmCrz* 5' upstream sequence specific forward primer (5'TGCTTTGGAATGCAGTTGAAATGTGG) and a reverse primer (5'CGGCAGAAGTTAGTCCTGTTTGCAGG) that is specific to the 5'- UnTranslated Region (UTR) of *D. simulans Crz* cDNA (accession no. AJ851891; Choi et al., 2005) and purified *D. simulans* genomic DNA as a template. We reasoned that genomic sequences between *D. melanogaster* and *D. simulans* might be highly conserved, as shown for the *Crz* gene (Choi et al., 2005). The PCR products were subcloned into the pGEM-T easy vector, and sequenced.

In order to identify *D. pseudoobscura Crz* gene (*DpCrz*), nucleotide sequence corresponding to putative CRZ peptide was searched in the *D. pseudoobscura* genome database using tBlastn program. This resulted in one sequence (Contig 815_Contig5737) that is highly homologous to the CRZ encoding sequence. The sequence was further analyzed to identify putative 5' upstream sequence.

Immunohistochemistry

The larval and adult CNS, including ventral nerve cord (VNC) was dissected and fixed in fixative (4% paraformaldehyde in 0.1M sodium phosphate butter with 7.5% saturated picric acid) for 30 minutes and rinsed in PBS solution containing 1% Triton three times for 15 minutes each. After incubating in blocking buffer (4% normal donkey serum in TNT (0.1 M Tris, 0.3 M NaCl, 0.5 % Triton X-100, pH 7.4) for 2 hours, primary antibodies applied and incubated overnight at 4°C. Anti-Crz raised in rabbit against synthetic peptide of 15 residues (VDPDPENSAHPRLSN) corresponding to part of Crz-associated peptide, was applied at a 1:300 dilution for the whole mount immunohistochemistry, as described previously (Choi et al., 2005). The CNSs were then rinsed in TNT 6 X 10 minutes each at room temperature. The Crz-immunoreactive signal was detected by FITC-conjugated secondary antibody raised in donkey (Jackson Immuno-Research).

Expression analysis

Each *gal4* transgenic fly was crossed to a *UAS-lacZ* (Brand and Perrimon, 1993; Phelps and Brand, 1998) or *UAS-mCD8GFP* (Lee and Luo, 1999) reporter lines. The progeny of each crossing were processed for X-gal histochemistry or green fluorescent protein (GFP) detection (Park et al., 2000). For adult expression, male progeny of each crossing were incubated for a week in fresh vials and then processed similarly. To perform X-gal histochemistry, dissected larval and adult CNSs fixed (0.2% glutaraldehyde in PBS, Sigma) for 5-10 minutes at room temperature. Subsequently the CNSs were rinsed 3 times in PBS for 5 minutes with gentle agitation. The rinsed

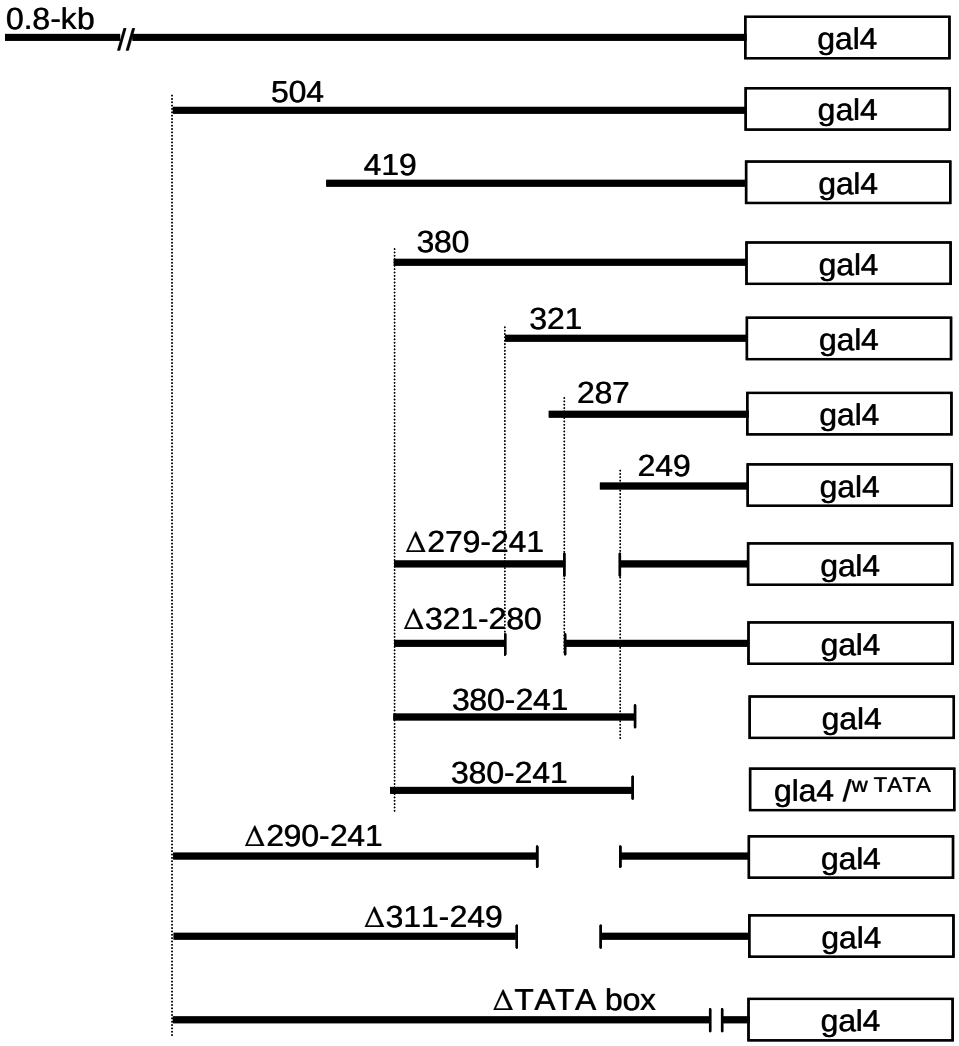

Figure 2-1. Schematics of *Crz* promoter-*gal4* fusion constructs for germ-line transformation. Serial 5'-deletions and deletions internal fragments are indicated by the nucleotide positions

CNSs were incubated in X-gal staining solution (10 mM Sodium phosphate (pH 7.4), 150mM NaCl, 1 mM $MgCl_2$, 5 mM Potassium ferrocyanide (P-9289, Sigma), 5 mM Potassium ferricyanide (P-3667, Sigma), 0.1% Triton X-100, and 1 mg/ ml X-gal) at 37℃ until color developed. To remove crystals, CNS was rinsed PBS, and 70% ethanol (EtOH) for 30 min. The CNSs were dehydrated in 70% and 90% EtOH for 10 min, cleared in 90% glycerol, and then mounted on slide. For GFP detection, dissected tissues were incubated in fixative (4% paraformaldehyde in PBS) for 30 min at room temperature. Subsequently the CNSs were washed 3X in PBS for 5 min each, cleared in 30% and 60% glycerol in PBS for 10 min. The cleared CNSs were mounted in vectashied (Vector Laboratory). At least 5 specimens of independent 3 lines from each *gal4* transgenic lines were tested.

Electrophoretic Mobility Shift Assay (EMSA)

In vitro interaction between *Crz*'s *cis*-acting regulatory site and its cognate binding factors was assessed by using a non-radioactive EMSA kit (Pierce). For this, putative *Crz* regulatory sequence (between -380 and -219) was labeled with biotin at its 3'-terminus by PCR using biotin-tagged reverse primer. Competitors were prepared similarly, except for an unlabeled reverse primer used (Table 2-1). Both nuclear and cytoplasmic protein extracts were prepared simultaneously from ≈ 60 CNSs dissected from wandering third-instar larvae (y w) as per provided protocols. In a binding reaction (20 µl), 10-fmol biotin-labeled DNA was incubated with ≈ 12 CNS-equivalent nuclear or cytoplasmic extracts for 20 min at room temperature. The reaction mixture was then

separated by 4% acrylamide gel electrophoresis and transferred onto a nitrocellulose membrane. The biotin-labeled probe was detected by incubating the membrane with HRP-conjugated streptavidin and fluorogenic HRP substrate according to the supplier's instruction. For a competition reaction, 200-fold unlabeled probe was included.

Phage display

To obtain Crz-specific transcription regulators, phage display was performed (Danner and Belasco, 2001). T7 phage cDNA library was constructed from the *eya*^{1} mutant fly head mRNA using a commercial kit (T7 Select System, Novagen). Briefly, total RNA was purified from *eya*^{1} adult heads, and then poly-A RNA was further purified by using Qiagen Oligotex kit. Approximately 500 ng of poly-A RNA was subjected to a reverse transcription reaction using random primer. The resulting cDNA was ligated with linkers (5' with *Eco*RI linker and 3' with *Hind* III linker), digested with *Eco*RI and *Hind* III, and then inserted into T7 phage vector. Following in vitro packaging, the T7 phages were amplified through infection with *E. coli* (BLT5403) strain, which resulted in 5 X 10^{9} PFU/ml. To check quality of the library, ten plaques were randomly chosen and subjected to PCR using T7SelectUP and T7SelectDOWN primers. The phage titer was determined by plaque assay. Briefly, 250µl of serial bacteriophage diluents (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7}) mixed with 500 µl of host *E. coli* strain (BLT5165). Then 2-3 ml of melted top agarose was added to the mix at 45-50℃ and contents were poured the standard Petri-dish and incubated 3-4 hours at 37℃. When plaques appeared, the incubation was stopped and counted. The 100 pM of biotin labeled probe,

previously employed for EMSA, was used as bait. The bait was immobilized to 1 mg of streptavidin-magnetic beads (Dynal Biotech) and washed 3X 5 min with 1ml B & W buffer (10 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 2 M NaCl). Biotin-labeled DNA probe was mixed gently with 1ml of washed magnetic beads, and incubated for 30 min at RT. The DNA probe-bead complex was washed 2X 5 min with 1 ml B & W buffer and 2 ml phage extraction buffer to remove unbound DNA probe. The T7 *eya*[1] brain cDNA library (1.5 X 10^{10} PFU/ 3 ml) was incubated with washed probe-bead complex for 1 hr at RT. The phage-probe-bead complex was washed 3X with 1ml phage extraction buffer. The eluted phages were amplified by plaque amplification method (Danner and Belasco, 2001, Fig 2-15) and then used for the subsequent rounds of screening to enrich specific phages displaying the putative *Crz* specific *cis*-acting binding proteins. After each round of screening, more than 18 randomly picked plaques were amplified to detect enrichment by PCR whether more homogeneous, and then phage DNA was purified by using a commercial kit (Wizard lambda kit, Promega). Total five rounds of phage display were performed. The purified phage DNA was digested with *Eco*RI and *Hind* III, and then the *Eco*RI/*Hind* III fragment was cloned into the *pBluescript* SK^+ vector, and then sequenced.

Affinity purification for *Crz* specific *cis*-acting elements

To fish out *Crz* specific *tans*-acting elements in larval stage, affinity purification was performed. For this, biotin-labeled putative *Crz* regulatory sequence and *y w* larval CNS nuclear and cytoplasmic protein extracts were prepared, as previously described in EMSA assay. In a 1X EMSA solution (10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 5%

glycerol, 1 mM EDTA, 4 mM DTT), 150-ng of biotin-labeled DNA and ≈ 25 CNS-equivalent extracts were incubated for 30 min at RT with 10 µg of sonicated salmon sperm DNA as a nonspecific binding blocker. During incubation, 200 µg of streptavidin-conjugated magnetic beads (Dynal Biotech) was activated by 2X wash for 10 min in 1X EMSA solution at RT. The DNA-protein mixture was immobilized by incubation with magnetic beads for 30 min at RT. The DNA-protein-bead complex was washed 3X 10 min with 1X EMSA solution. After washing unbound proteins, 1X EMSA solution was discarded and then the mixture resuspended was using 20 µl of 1X SDS-protein loading dye. The samples were incubated at 100℃ for 10 min and then separated by 10% SDS-PAGE gel. Putative *Crz* specific *trans*-acting elements were detected by Coomassie staining.

IV. RESULTS

Distinct regulatory regions for *Crz* expression in the larval CNS

Crz is normally expressed in three groups of neurons in the larval CNS, namely DL, DM, and VNC neurons (Choi et al., 2005). Previously, 1.2-kb sequence upstream of the *Crz* gene was demonstrated to faithfully recapitulate endogenous *Crz* gene expression in the larval CNS (Choi et al., 2006). Since the 1.2-kb upstream sequence regulates tissue- and developmental stage-specific *Crz* expression, and *Crz* expression occurs in distinct groups of cells, we were interested in identifying separate *cis*-acting regulatory element(s) for each group of cells in vivo. For this, we performed 5'-promoter bashing experiments in which serial 5' deletions of 1.2-kb *Crz* promoter were made (Fig.

2-1). These shorter fragments were fused to *gal4*-endcoding sequence and used for germ-line transformation. Instead of *lacZ* or *GFP*, we chose *gal4* as a reporter because the *gal4-UAS* trans-activation system (Brand and Perrimon, 1993) is more sensitive to detect weak promoter activities due to amplified production of the reporter proteins (cf. Park et al., 2000). In fact, the data confirmed that GFP signals derived from the homozygous *Crz-GFP* transgene (i.e., direct fusion between *Crz* promoter and *GFP* coding sequence) were noticeably weaker than signals obtained from flies carrying one copy of the *Crz-gal4* and *UAS-mCD8GFP* transgene (Allgyer and Park, unpublished result). Furthermore, as we hoped, if any *gal4* construct drives expression in a specific subset of *Crz* neurons, then this *gal4* driver would be a valuable tool for investigating neuronal group-specific functions by employing various types of transgenic manipulations (e.g., Kaneko et al., 2000; Lee and Park, 2004).

Crz-gal4 transgenic lines were crossed to a *UAS-mCD8GFP* or *UAS-lacZ* reporter line and the progeny processed for GFP or lacZ expression patterns, respectively. Reporter expression driven by the 802-bp as well as 504-bp upstream fragments was identical to endogenous *Crz* expression patterns in the third-instar larval CNS (Fig. 2-2 A-C). To find minimal driver, we tested shorter upstream fragments, 287- and 249-bp (Fig 2-1). The 249-bp fragments fail to generate reporter gene expression (Fig. 2-2 F), while the 287-bp fragment produced variable the DL neurons (from one to six) and vCrz (Fig 2-2 D and E, Table 2-2). This variation showed among lines as well as specimens within the same line. In addition, levels of expression were in general very weak and axonal projection was hardly detectable (Fig 2-2 D and E). Therefore,

these results indicated *Crz* regulatory region exists between -249 and -504 bp upstream sequence.

To identify cell-specific regulatory regions, we performed further 5´-promoter bashing experiments. Interestingly, reporter expression mediated by the 419-bp upstream sequence was observed in the DL and vCrz neuronal groups, but not in the DM group (Fig. 2-3 B, E and H). Co-labeling with anti-Crz (Fig. 2-3 A, D and G) further verified the lack of GFP signals in the DM neurons (Fig. 2-3 C, F and I arrowhead). The absence of DM expression by the 419-bp upstream sequence suggests that the sequence between -504 and -419 (we refer to this interval as -504::-419 and similar designations were used for other genomic intervals) is essential for DM-specific expression, while responsible regulatory sequence(s) for DL and vCrz expression is located within the 419-bp upstream region. Since our further analyses of this region imply that regulatory elements for the DL and vCrz neurons are dissimilar, we describe the results separately for these two neuronal groups.

Multiple *cis*-acting elements for DL-specific *Crz* and two *cis*-acting elements for vCrz expression

The existence of a DM-specific regulatory region prompted us to investigate further whether there are separate *cis*-acting regulatory sites specialized for DL- specific *Crz* transcription. In contrast -287-bp upstream driver, the reporter gene expression driven by the 321-bp upstream region showed invariable DL (Fig. 2-4 B, E and I). Therefore, a simple interpretation of these results is that the DL-specific regulatory sequence is positioned in an interval of -321::-249. However, vCrz expression driven by

-321-bp upstream region is highly variable. After nine independent lines were tested to evaluate this construct, we subcategorized three different groups. For example, S5a

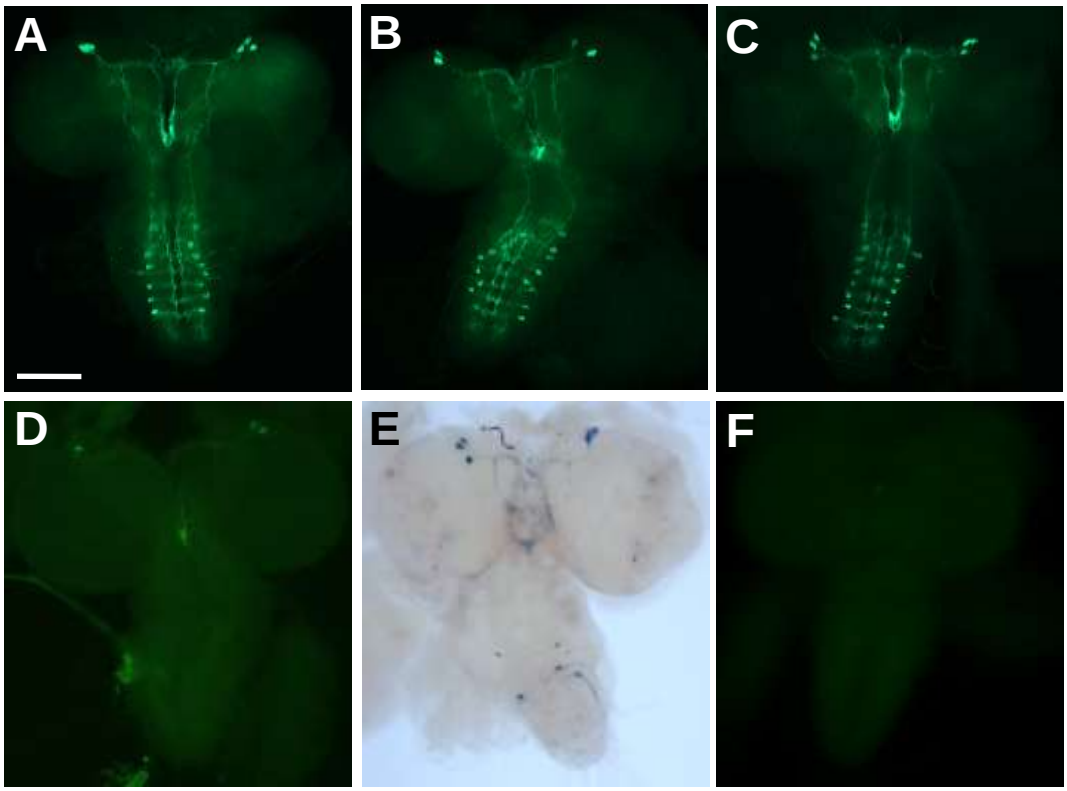

Fig. 2-2. Representative reporter gene expression by 5' truncated *Crz* promoter driver. (A) 802bp-upstream, (B, C) 504bp-upstream T1a (B) and T2b (C) lines. Both 802 and 504bp-upstream drivers show indistinguishable signals compared original 1.2kb-upstream driver. (D, E) Reporter gene expression driven by 287bp-upstream. Reporter gene shows only few numbers of DL and vCrz neurons. Representative image shows from X2 (D) and S5 (E). (F) No reporter gene expression driven by 249bp-upstream driver. Scale bar indicates 100µm

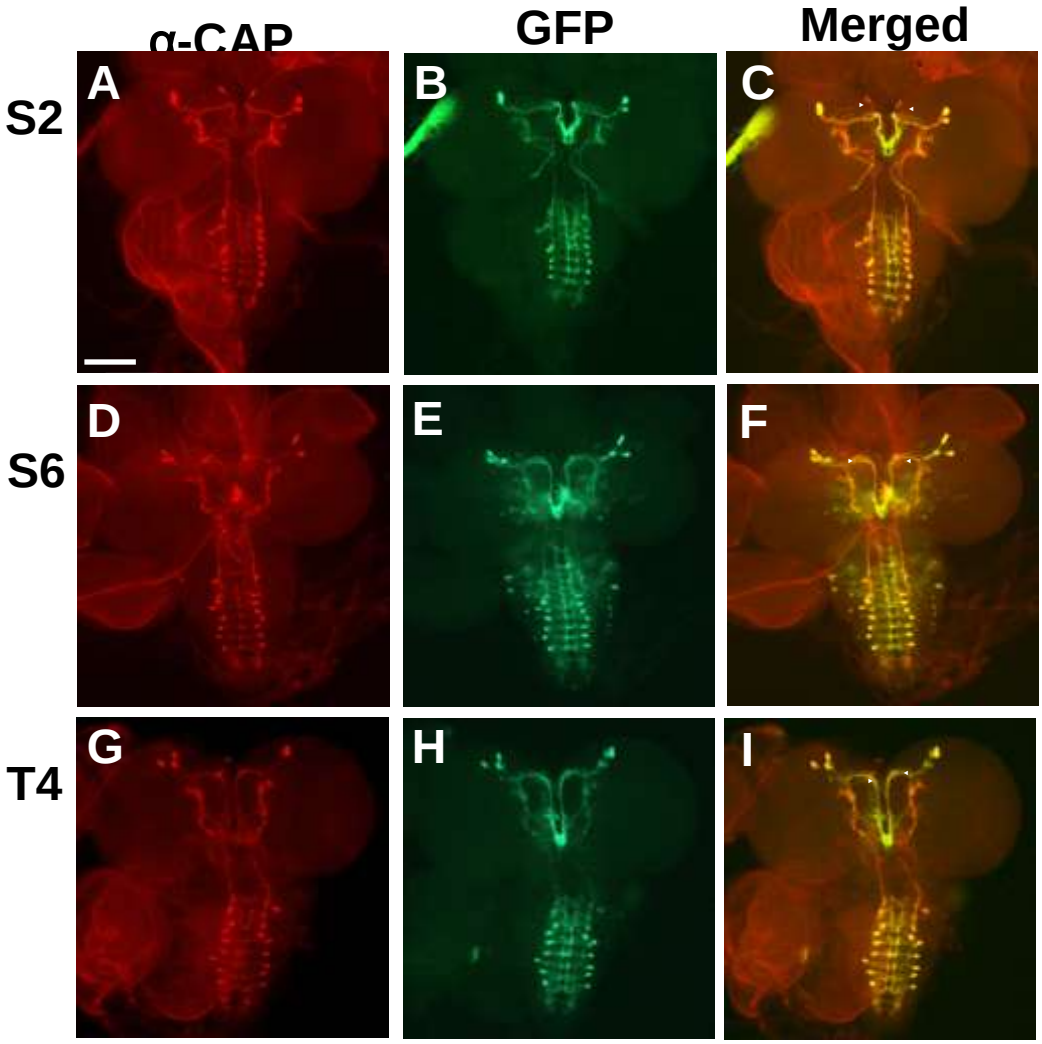

Fig.2-3. Representative reporter gene expression by -419 Crz promoter driver. Three independent lines are shown here (S2, S6 and T4). (A, D and G) Crz immunoreactive images. (B, E and H) mCD8GFP expression. (C, F and I) Superimposed image between Crz immunoreactivity and reporter gene expression. All signals are colocalized on DL and VNC neurons, but DM neurons are only Crz immunoreactive (arrowhead). In S6 line, some ectopic signals existed (E). Scale bar indicates 100µm

line showed only one vCrz neuron (Fig. 2-4 B). This GFP expressed neuron is verified as a vCrz neuron by co-labeled with anti-Crz (Fig. 2-4 C). In case of the T2a line, all 8 pairs of vCrz cells were clearly visible (Fig. 2-4 E-G). In T6 line, GFP showed intermediate expression between S5a and T2a. Only 3-6 pairs of vCrz cells produced reporter gene expression (Fig. 2-4 H-I). Therefore we subgrouped these lines: weak (S3a, S3b, S5, and T7), intermediate (X1a, X1b, S2b, and T6), and near normal (T2a, Table 2-2) groups.

Since spatial *Crz* expression patterns in the juvenile CNSs are consistent among other *Drosophila* species--*D. erecta*, *D. simulans*, and *D. virilis* (Choi et al., 2005), we reasoned that functionally important regulatory sequences within -321::-249 might have been selected during the course of evolution. To identify such conserved sequence motifs, the *D. melanogaster Crz* upstream sequence was compared with corresponding sequences from three other *Drosophila* species, including *D. simulans*, *D. virilis*, and *D. pseudoobscura*. Alignment of these sequences revealed four blocks of highly conserved regions (Fig. 2-5). The first is a 50-bp stretch at -290::-241; the second, a 35-bp sequence at -172::-138 interval; the third, a consensus TATA box; and the fourth, initiator. The latter two elements are known for binding of basal transcriptional machinery Arkhipova, 1995; Smale, 1997). The second sequence (-172::-138) is unlikely to play a role for the group-specific *Crz* expression, since the 249-bp fragment including this region was incapable of directing reporter expression in any of Crz neurons (Fig. 2-2 F).

To evaluate regulatory roles played by a more distally located sequence (-290::-241), which is part of previously defined interval at -321::-249, a 504-bp fragment

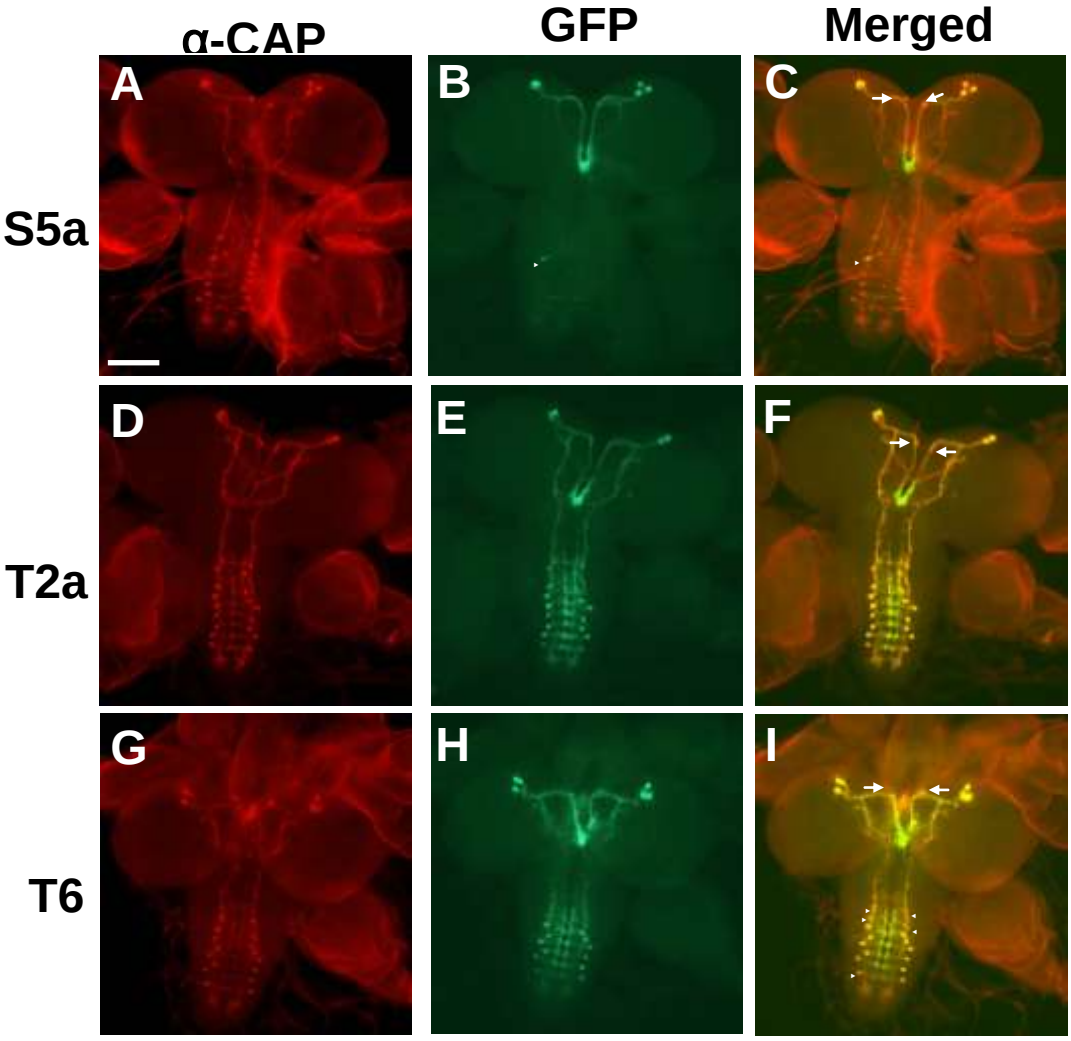

Fig. 2-4. Representative reporter gene expression by -321 Crz promoter driver. Three independent lines are shown here (S5a, T2a and T6). (A, D and G) Crz immunoreactive images. (B, E and H) mCD8GFP expression. (C, F and I) Superimposed image between Crz immunoreactivity and reporter gene expression. All DL neurons show colocalized signals, but only DM neurons are Crz immunoreactive (arrow). In the VNC region, reporter gene expression patterns are variable. In S5a line, very weak signals are detected in the VNC (B and C. arrowhead). In contrast, T2a line shows 8 pairs of VNC neurons (E and F). T6 line, 4-5 neurons do not expressed reporter gene on the VNC (I. arrowhead). Scale bar indicates 100µm

lacking this region was generated and tested for its promoter activity ($504^{\Delta 290\text{-}241}$; Fig. 2-6 B and C). Significantly, reporter expression driven by this construct was detected in all DL neurons (Fig. 2-6 C), suggesting that this conserved sequence is either dispensable or redundant with respect to directing DL-specific expression. When deletion was extended an additional 21-bp of 5' upstream ($504^{\Delta 311\text{-}249}$), the number of DL neurons displaying reporter expression was reduced by one to six cells, as were expression levels (Fig. 2-6 A and B). This result was comparable in part to that of 287-bp promoter (Fig. 2-2 D and E). Based on the results obtained using these two internally deleted constructs, it is likely that the sequence at 321::-291 is essential for DL-specific expression. However, since 287-bp is still able to drive, albeit weakly, reporter expression in the DL neurons, we predict that the -321::-291 region plays a dominant role, while the proximal -287::-249 region is still necessary for full activity in the DL neurons. To our surprise, however, the deletion of such a putative dominant element from the 380-bp upstream sequence ($380^{\Delta 321\text{-}280}$) did not affect DL-specific expression (Fig. 2-6 G), nor did another internal deletion ($380^{\Delta 279\text{-}241}$) (Fig. 2-6 D-F). These results suggest that an additional region located at -380::-322 might be functioning redundantly for DL expression, thus compensating for the loss of element at -321::-291. Taking all of these results into account, we propose at least three *cis*-regulatory motifs for DL- specificity: DL-d at -380::-322, DL-c at -321::291, and DL-p at -279::-249 (summarized in Fig. 2-8). Our data further suggest that at least two of these elements are required for normal *Crz* expression in DL neurons.

It is apparent that molecular components regulating *Crz* transcription in the DL neurons differ from those in the vCrz neurons, because several *Crz-gal4* constructs

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D. s -512 TTGTAAC-CTTATAACT-T-AA-TTAAAGCCTTTAGCTTTGCTCTGGAAAATGCTAT -462
D. m -503 TTGTAACTAGTCCACATAT-AACTTAATTAAAGTC--------GT-----TGCTATT -461
D. p -515 GCGAGATAACTAGTC-TTTGAA-TCAATTAAAACCCATCCGTGTTGCATGTGTATAT -483
D. v -550 AGCAGTTACCCTGTGCCACCATGGACATGTGGATATGACCATGCTGAGCGACGCGCC -504
                            *     *                 *


D. s -461 TTTT-GGCCGAG----CTTGTCTGCAAATCCACTTACCTGAGGTCAT-CC---AAGT -414
D. m -460 TT-TGGCCGAGTCCA-----TTTGCAAATCCATTTACCTGAGGTCAT-CC---AAGT -414
D. p -482 TT-TTTCTATTTTTG-TATATTTATAAAGATGTTTATTTTGGTAAATCCCCGCAAAT -428
D. v -503 GGATTCTGATCAGGAAAATA--TAAATCGTC-TTC---T-GCCTGTTACAT--ACAT -456
             *                      *       *   *       * *    *  *


D. s -413 CTAATTTGTACATTTTACT-C--CC-------TTTTGGAGTGGGAAGAAAATTA--C -368
D. m -413 CTAATTTGGACATTTTACT-C--CC------CTTTTGGAGTGGGAAGAAAATTA--C -368
D. p -427 CAAATTCCCTGATAACAGAGCAGCCAAAGGTCTTCCCGAGCCTTCTTCAATCCAGCC -371
D. v -455 TTGCACAGAAACATTAAAT-CT--C----GATTTGGCCATTTGCAATGCGTACAGGG -406
                         *   *   *       **    *             *

D. s -367 TTTTTCGGC-----TCA-GCATTTTATTTTAAG---CCCTTTGCCTTTTGCCTTTTG -319
D. m -367 TTTTTCGGC-----TCAAGCATTTTATTTTAAG---CCTTTTGCCTTTTGCCTTTTG -319
D. p -370 CCCTCCGGCAGTGGCCACAAATTGCTTTTCGGGGGAGCAATTTTAGTTTATCTTCTG -314
D. v -405 TA-TCCGAACAGATGCCCTAACC-C-TTTCACTGTTGCTGTTGTGCTCTGTTTTCCG -352
             * **                   ***           **    * *   **  *

D. s -318 CCACC-CACTTTCTGATTTTGCCCGGG-AA-AAGTGGTAGTTTTCCAATCAGCAAAT -265
D. m -318 CCACC-CACTTTCGGATTTTGCCCGGG-AA-AAGTGGTAGTTTTCCAATCAGCAAAT -265
D. p -313 CCACGGCAGAGGCGTGTGAGAGCCTTTCAACAAGTGGTAGTTTTTCAATCAGCAAAT -257
D. v -351 ACAGAGTCCTGGCGCA---GGACTCTGGAGCAAGTGGCAATTTTCCAATCAGCAAAT -298
           **         *         *     *  ****** ****** ************

D. s -264 GCATTCAGTTTGAGCAGCCAGCTGTCGTTGGCACAAGGGGCGG-TAGGGGGAGCTGG -209
D. m -264 GCATTCAGTTTGAGCAGCCAGCTGTCGTTGGTACAAGGGGCGG-TGGCGGCAGCTGG -209
D. p -256 GCATTCAGTTTGAGCAGCCAGCTGGAGC--GTACTGGGCGTGGGTTGTGG--GTTGG -204
D. v -297 GCATTCAATTTGATCAGCCAGCCGGAGT-------------GG-TGGGCGTGGCACG -254
          ******* ***** **********  *              ** * *  *  *   *

D. s -208 GCGTGGCCAC-AGATGTGGGCGCTAACTGCCAAGTTGAAACAATTGGGTGCTAATAA -153
D. m -208 GTGGCCAC-GGGCATGTGGGCGCTAACTGCCAAGTTGAAACAATTGGGTGCTAATAA -153
D. p -203 GGGCCCCGACTTTATGTGGGTGTTAACCACAGCCTTAAAACAATTGGGTGCTAATAA -147
D. v -253 TGGG-T--ACTAAATGCCATC-TTGGCCAC-GCATGAAAACAATTGCCAACTAATAA -202
                      ***       *  *  *    *  ********* *  *******

D. s -152 TCCCCGGTTAATCTGGGTAAGTGTGTGTTTCTGGAGGGCATTGAAGCAAGGATTCTG  -96
D. m -152 TCCCCGGTTAATCTGGGTAAGTGTGTGTTTCTGGAGGGCATTGAAGCAAGGATTCTG  -96
D. p -146 TCCCA-GTTAATCTGCATAAGTGAGAGC----GGGTGT-ATGGCTGGAAGGCAGCGG  -86
D. v -201 TCTCA-GTTAATCTGCATGTGTGCG-GCGCCTGGAGGGCATCT-TGCCAGGAAAA-G -149
          ****  ************  *** * *     **  *  **    *  ***     *

D. s  -95 GCGGAAA------A--C-GCACA---CA-CA--TCAC--------------------  -74
D. m  -95 GAGGAAA------A--C-GCAGA---CA-CA--TCAC--------------------  -74
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D. p  -85 -AGCACAAA--TTA-CCAGCA-----CATCC--TT----------------------  -62
D. v -148 CACTTGTTGCCACACCCCGCACTCGGCACCCCCTTATCCATATCTATTCACCCCTTT  -92
                       *  * **      ** *   *

D. s  -73 ---G----------CA-T-CC------TT-TG-GCAACCA-TCC-TTTACGGCTATC  -42
D. m  -73 ---G----------CA-T-CC------TT-TG-GCATCAA-TCC-TTAATGGCTATC  -42
D. p  -61 ---GG---------AAGTAG-------TTCTCTGCTCTGC-TCT-TTGATGGC-ATC  -27
D. v  -91 TTTGGAGCCACGTTTATGGCCGTGGGATTTT-AGCGAAATTGCAGTTTGCTCCTTTG  -36
              *             *             ** *  **            **      *  *

D. s  -41 -CAACATCGATATAAAAGCCCAT-CGAGGAGCATCAAT-AG-TTCAGACG  5
D. m  -41 -TAACAACGATATAAAAGCCCGT-CGAGGAGCATCAAT-AG-TTCAGACG  5
D. p  -26 --A------ATATAAAAGCCC-T---GGCAGGA-------CCCTCAGACG  5
D. v  -35 GT--------TATAAAAGCGGCTGTCAGCAGCAATAA--AGGCTCAGACG  5
                    *********   *    * ** *          *******
```

Fig. 2-5. Sequence alignment of *Crz* promoter sites of four *Drosophila* species. Red boxes indicate the TATA box. Yellow boxes represent the highly conserved region for basal *Crz* expression. Magenta boxes represent another conserved region for potential DL expression. This region conserved 46 out of 50 bps among *Drosophila* species. Asterisks indicate the identical bases in all species, and dashes for gaps are inserted with no homology region. *D. s*: *D. simulans, D. m*: *D. melanogaster, D. p*: *D. pseudoobscura* (Contig815_Contig5737) and *D. v*: *D. virilis* (scaffold_53:348198-348777). Identify: 92% (*D.m*. vs *D. s*), 40.2% (*D.m* vs *D. v*.)

described above were sufficient for DL expression, but insufficient for vCrz expression (Fig. 2-4 and 2-6 A-F).

The 419-bp fragment drove reporter expression in all of the vCrz neurons (Fig. 2-3); however, the 321-bp showed highly variable results among transgenic lines, as well as among specimens taken from the same line (Fig. 2-4). Such variability is likely to reflect the fact that the 321-bp sequence contains incomplete vCrz enhancer, which seems to be highly sensitive to the genomic environment of the insertion sites. Near-absence of GFP signals in the vCrz neurons (S3a, S3b, S5, and T7, Table 2-2) could be due to a mutational effect caused by a transgene inserted at a certain locus; however, this does not seem to be the case, because endogenous *Crz*-immunoreactivity was intact (Fig. 2-4 A). A shorter 287-bp upstream fragment induced lacZ expression very weakly in only a few vCrz neurons (Fig. 2-3 D and E), while 249-bp fragment failed completely to drive reporter expression (Fig. 2-3 F). Taking these results together suggests that regulatory sequence(s) for vCrz expression is mapped approximately in a region between -419 and -249.

Of interest, three constructs, one devoid of the -290::-241 region ($504^{\Delta 290\text{-}241}$), another eliminating -249::-311 ($504^{\Delta 311\text{-}249}$) and the other deleting -279::-241 ($380^{\Delta 279\text{-}241}$), directed reporter expression similarly in reduced numbers of the vCrz neurons(Fig. 2-6 A-F). These findings, together with previous data, suggest that sequence of -279::-249 is required for optimal vCrz expression. In addition, since the 321-bp construct is also insufficient for vCrz expression (Fig. 2-4), it is conceivable that there is an additional regulatory element(s) within the -380::-322 region. Perhaps these separate elements function cooperatively to produce appropriate vCrz expression.

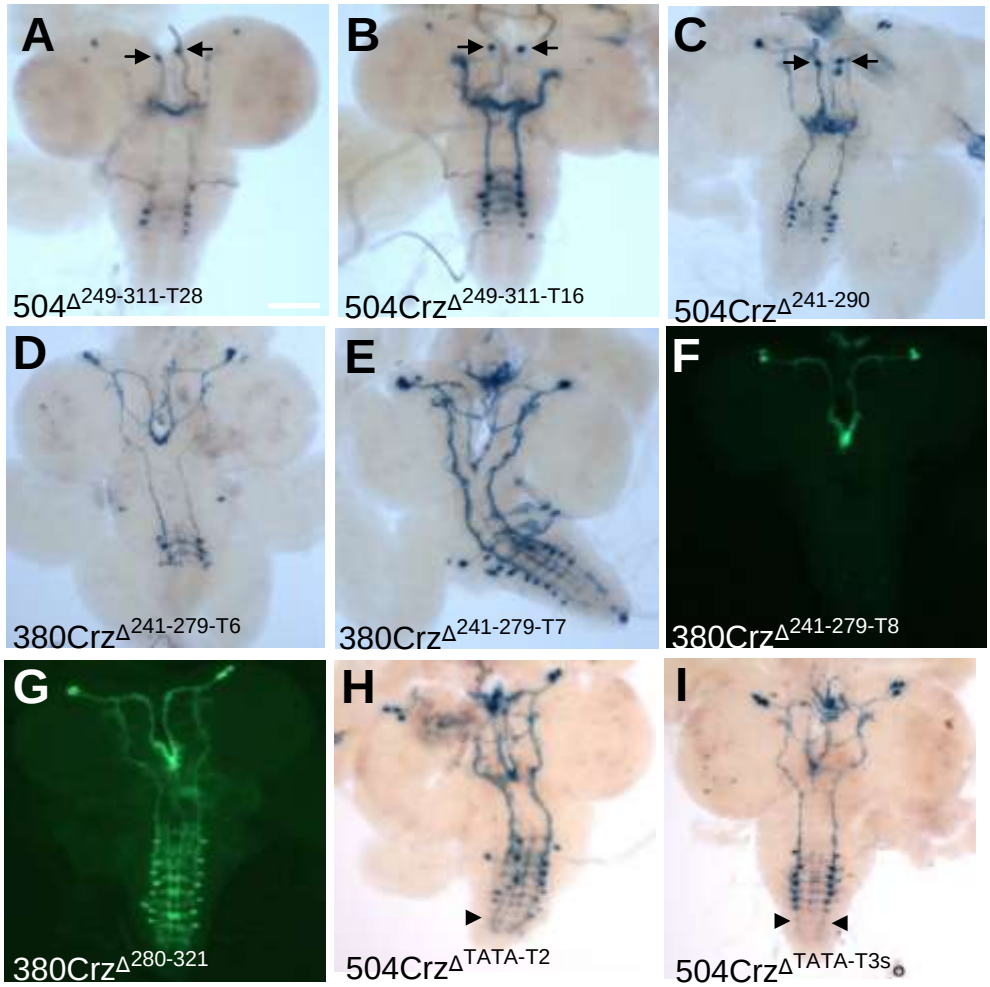


Fig. 2-6. Representative reporter gene expression by internal deletion drivers, as indicated in each panel. The drivers induced reporter gene expression in DM neurons (A-C; arrow), however, the drivers partially drove reporter gene expression in the VNC. (G) Normal DL and VNC expression driven by internal deletion between -280 and -321 bp upstream. (H and I) Reporter gene expression driven by 504-bp sequence lacking TATA box. The missing region of *lacZ* expression in the VNC are indicated by arrowhead. Scale bar indicates 100µm

Therefore, we refer to the distal -380::-322 region as vCrz-d and the proximal -279::-249 region as vCrz-p (Fig. 2-12). In support of this prediction, a deletion of the region between the two elements ($380^{\Delta 321\text{-}280}$) did not affect normal vCrz expression (Fig. 2-6 E), further verifying that this middle region is dispensable for appropriate control of *Crz* transcription within the vCrz neurons.

To identify roles of the DL and vCrz specific *cis*-acting element, we tested two DL and vCrz upstream fragment only constructs ($380\text{-}241^{\Delta 321\text{-}280}Crz^{TATA}$ and $380\text{-}241Crz^{TATA}$). Even the basic transcription factor binding site TATA box was supplied by *gal4* vector, these DL and vCrz specific upstream fragments failed to express reporter gene or produced very weak in DL and vCrz (Fig. 2-7). These results indicated that sequence within 240-bp upstream region is still essential perhaps for basal transcriptional activity.

In summary, transcriptional regulation in the vCrz neurons requires two separate regulatory sites; a proximal one at -279::-249 (vCrz-p) and a distal one at -380::-322 (vCrz-d), which overlap approximately with DL-p and DL-d, respectively (Fig. 2-8). Despite such overlap, the transcriptional regulatory mechanisms for vCrz expression are unlikely to be the same as those for DL neurons. This notion is buttressed by the fact that several of the promoter constructs tested here induced reporter expression differentially between the vCrz and DL neurons (Fig. 2-4 and 2-6).

Role of the TATA box

Although a consensus proximal promoter element, termed TATA box, is important for core promoter activity in a number of genes and the presence of the

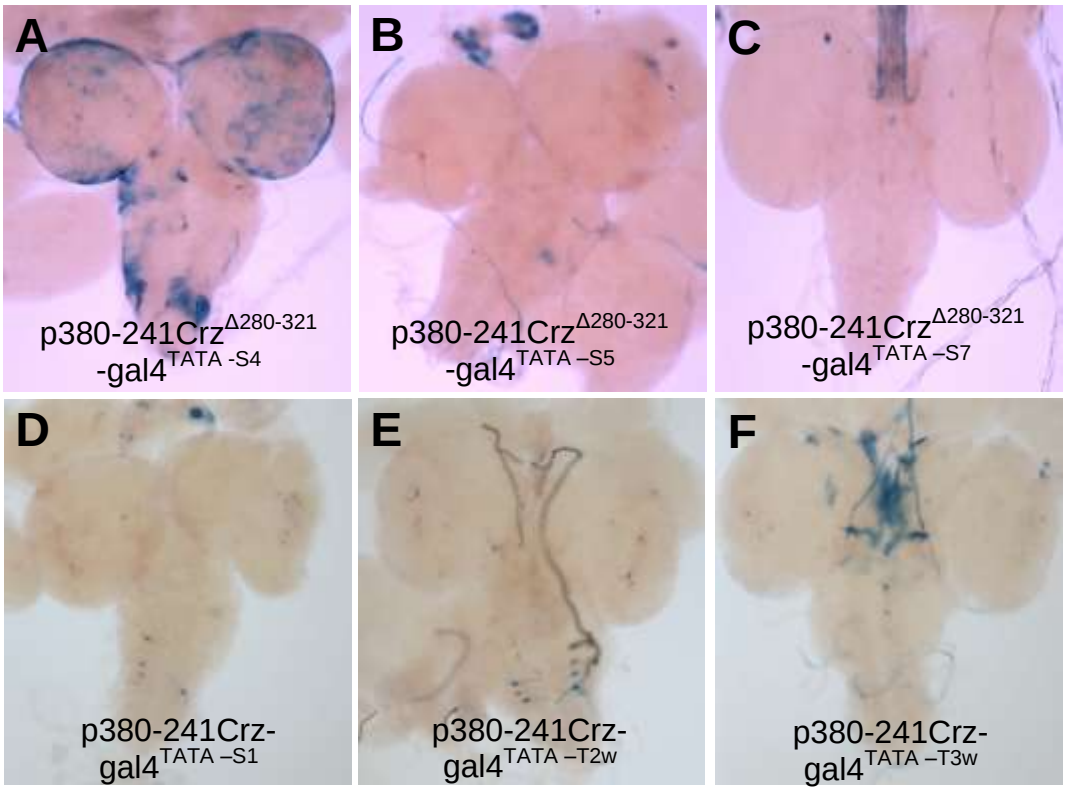


Fig. 2-7. DL and VNC *cis*-elements only are not sufficient to drive DL and vCrz expression. (A-C) internal deletion between -280 and -321bp regions. (D-F) 380-241 region only construct. Scale bar indicates 100µm.

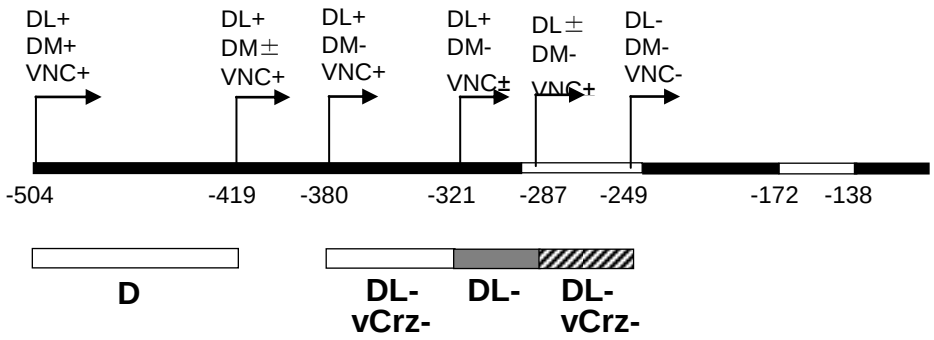


Fig. 2-8. Schematic diagram of putative *Crz cis*-acting element in larval stage. The numbers indicate the position of 5' upstream region of *Crz* promoter. The blue region of *Crz* promoter represents the highly conserved region among the *Drosophila* species. The (+) means that the reporter gene expressions showed like wild type. The (±) means that the reporter gene expressions showed reducing the cell numbers or missing the some parts. The (–) means that the reporter gene expression did not show.

TATA box is evident in several *Drosophila* neuropeptide genes including *Pdf* (Park and Hall, 1998), *npf* (Wu et al., 2003), *FMRFamide* (Taghert and Schneider, 1990) and *Adipokinetic* hormone (Alcorta, 1991; Noyes et al., 1995; Lee and Park, 2004), functional analysis of this element has not been done. To test the roles of the TATA box for the transcriptional control of the *Crz*, a 504-bp construct lacking TATAAA ($504Crz^{\Delta TATA}$) was tested (Figs. 2-6 H and I). Since the TATA-box is known to be essential for binding of basal transcriptional complex containing RNA polymerase II (reviewed in Smale, 1997), we expected no expression whatsoever driven by this mutant construct. Quite contrary to this prediction, four independent transgenic lines tested showed normal lacZ reporter expression in the DL and DM neurons (Fig. 2-6 H and I). In the VNC, *Crz* expression was marginally affected; only 1 or 2 neurons in the last pair of the vCrz neurons lacked lacZ expression (arrowheads in Fig. 2-6 H and I). This finding suggests that the TATA box is not vital for Crz transcription in most Crz neurons. This result further suggests diversity of the transcriptional regulatory mechanisms even among vCrz neurons.

Sex-specific *Crz* expression in adult CNS

In adult stage, we reported that *Crz* mRNA was expressed 6-8 cells in dorso-latero-posterior (hereafter we called DLP) region in each hemi-brain and medulla region. However, the transcripts of *Crz* in medulla were not translated. These *Crz* expression patterns showed non-sex specific manner (Choi et al., 2005).

In this study, we did further analysis of *Crz* expression pattern in the adult CNS. The cell numbers and axonal projections of Crz-immunoreactivity in the brain showed

indistinguishable between male and female (Fig. 2-9 A and B). Interestingly, male tissues showed two pairs of Crz immunoreactive cell in the abdominal ganglion, while such signals are not detected in the female (Fig. 2-9 C and D). This result suggests that the sex-determination genes might control the male specific *Crz* expression, we reported for male-specific *npf* neurons (Lee et al., 2008).

Adult specific *cis*-acting element

Transcripts of the *Crz* gene were detected in three different regions in the adult male CNS; 6-8 DLP neurons, medulla, and four male-specific in the abdominal ganglion (ms-aCrz) neurons. As demonstrated in larvae, we hypothesize that the adult specific *Crz* expression in each subgroup is regulated by distinct *cis*-acting elements. To test our hypothesis, various *Crz-gal4* transgenic lines, previously described for larval expression, were examined for adult *Crz* expression.

As in larvae, 504-bp upstream was sufficient to produce the *Crz* gene transcripts in all, groups of *Crz* neurons in the adult. Reporter gene expression driven by 419-bp, and 321-bp upstream fragments were identical to that by 504-bp in adult CNS (Fig. 2-10 A-C, and data not shown for the 504-bp and 419-bp constructs). The levels of expression and axonal projections were indistinguishable among the constructs and inter-lines bearing the same construct. The results suggest that 321-bp upstream region is sufficient for adult *Crz* expression The 249-bp constructs failed to produce any reporter gene expression as in the larval stage (Fig. 2-10 D and E). By comparison, reporter gene expression driven by the 287-bp upstream construct was found in the medulla and ms-Crz neurons. However, only 1-5 cells were detected in

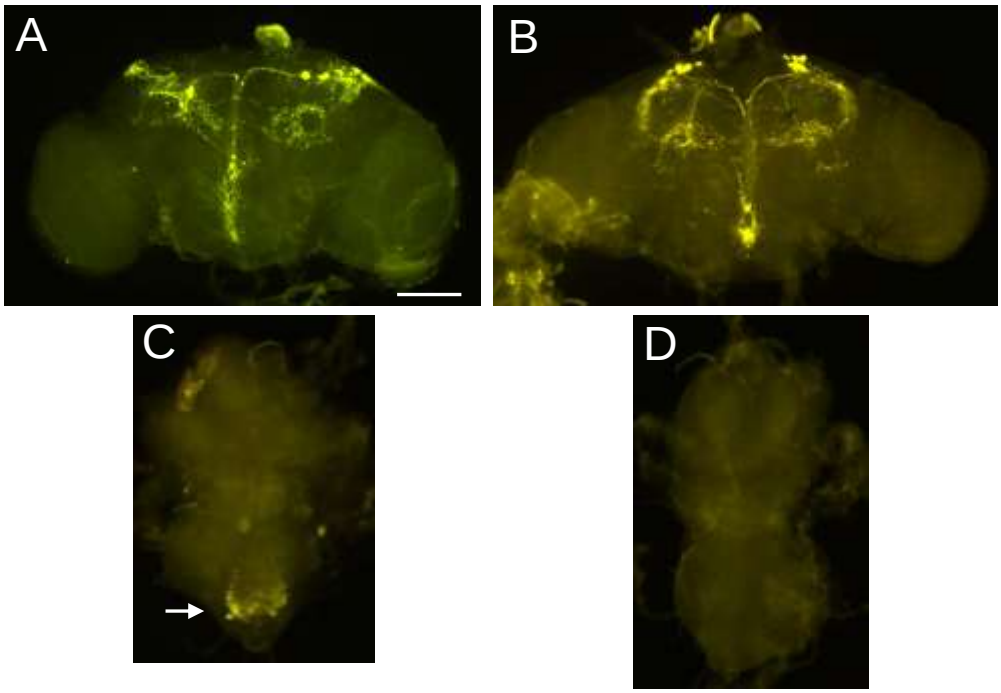


Fig. 2-9. Sexual dimorphism of *Crz* expression in adult. *Crz* expressing cells are indistinguishable in the brain between male (A) and female (B). Six to eight DLP cells were expressed in each hemi-brain. (C) Two pairs of CRZ-immunoreactive cells in the male abdominal ganglion (arrow). (D) Crz expression cells are blank in the female VNC. Scale bar indicates 100 µm

DLP region in each hemi-brain and the neuronal projections were only weakly detected (Fig. 2-11). In the larval stage, 287-bp construct drove expression weakly in variable number of DL neuronal *Crz* expression. Therefore, we propose that the adult specific *Crz cis*-acting elements are located between -240 and -321-bp region, which includes the highest conserved part in the Crz upstream region among *Drosophila* species (Fig. 2-5).

To further refine adult specific *cis*-acting elements, we investigated a construct lacking conserved region ($504^{\Delta 241\text{-}290}$). In the larval stage, the reporter expression was seen in reduced cell numbers and projections in DL and vCrz neurons. During metamorphosis, DL cells cell numbers increased from 3 to 6-8 per each hemi-brain (Choi et al., 2005). Thus the DLP neurons could be subdivided two groups according to their origin: DL-DLP neurons, which originated from larval stage, and pDLP neurons, which produced during pupa stage. As described previously, DL-DLP neurons projected their neurite into two different paths. One is median track then turned ventrally to reach the esophagus foramen and the other is posterior lateral track (Choi et al., 2005; Lee et al., 2008). Interestingly, the reporter gene expression driven by $504^{\Delta 241\text{-}290}$ construct detected only DL-DLP cells, and the ms-aCrz expression was undetectable (Fig. 2-12). Moreover, in the medulla region, reporter gene expression was variable; for example, T3 line produced signals, but S2 line did not. In the T3 line, the reporter gene was strongly detected in DL-DLP and their axonal projection (Fig. 2-12 A and B). However, in the S2 line, the reporter gene was found in DL-DLP cell body and very weak axonal projections in the dorso-lateral part and esophagus region (Fig.

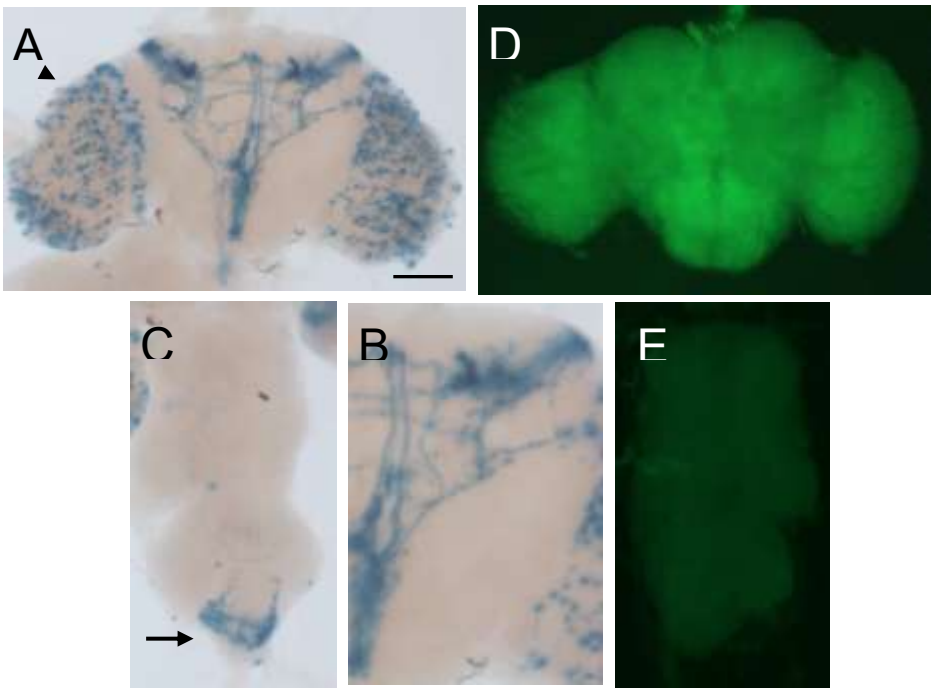


Fig. 2-10. Reporter gene expression by various 5' truncated promoter. (A-C) lacZ expression driven by 321 upstream sequence. The expression is evident in DLP and optic lobe (A. arrow head). Male specific abdominal ganglion expression in VNC region (C, arrow). Enlarged DLP expression in the brain region. Six to eight cells were clustered in each hemi brain and showed neurite pattern (B). (D-E) No reporter gene expression driven by 249 upstream sequence in the brain (D) and VNC (E). Scale bar indicates 100 µm.

2-12 D, arrowhead and arrow). The result strongly supports that the fragment between -250 bp and -290 bp harbors pDLP and male-specific *cis*-acting elements (Summarized in Table 2-3 and Fig. 2-13)

Putative *trans*-acting factors

Because transcriptional activation of genes results mostly from specific interactions between *cis*- and *trans*-acting regulators, we performed an EMSA to ascertain whether the *Crz* regulatory sequence identified above provides a binding site for putative trans-acting molecules. In doing so, nuclear proteins extracted from the CNSs of wild-type third-instar larvae were incubated with the biotin-labeled probe (-380::-219) that contains *cis*-acting sequences responsible for *Crz* expression in both DL and vCrz groups. Neither the probe alone nor incubation with cytosolic protein extracts, affected the electrophoretic mobility of the probe (Fig. 2-14). Significantly, incubation with nuclear proteins caused the probe to be shifted to an elevated position (arrow in Fig. 2-14). Since mere 0.2% of neuronal cells are likely to produce binding factors, it is not surprising that the shifted band was detected with only weak intensity. Importantly, such a shift was nullified by the addition of an excessive amount of unlabeled competitor probe (lane 3 in Fig. 2-14), thus pointing to the specific molecular interactions between the DNA probe and its cognate binding factors.

To find *Crz* specific transcription regulator, the phage display was performed (Fig. 2-15). This method has broadly been used to characterize interaction between *cis*-acting element and transcription regulators, RNA-protein interaction, protein-protein binding, and drug development (Danner and Belasco, 2001). After each round of

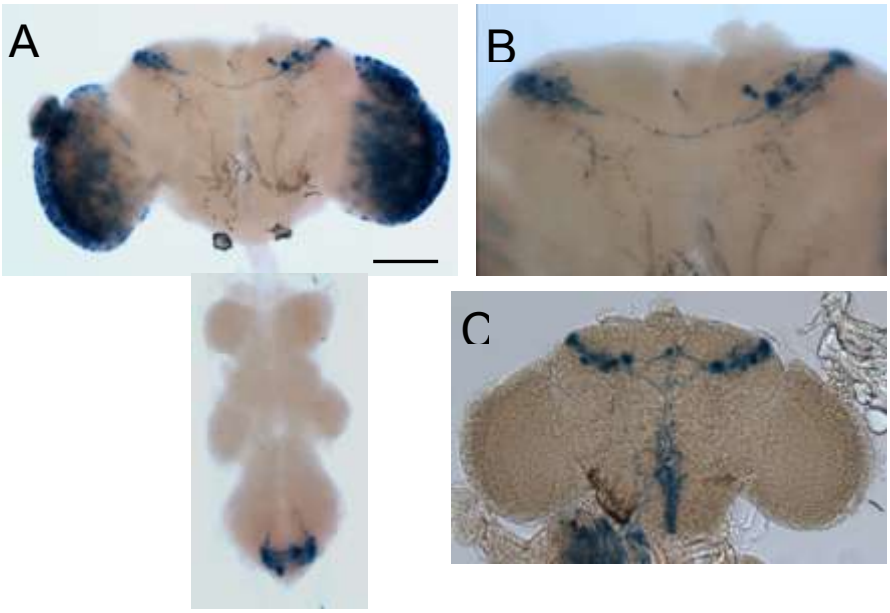


Fig. 2-11. Reporter gene expression by 287 upstream sequence. (A) Representative image of lacZ expression showed in DLP and optic lobe in brain region. Male specific abdominal ganglion expression showed VNC (S5). (B) Only 1-5 cells were detected with reduced projection. (C) Representative image of another line (T3) in adult brain and optic lobe. In contrast to S3, T3 line showed median projection at the subsophageal region. Scale bar indicates 100 µm.

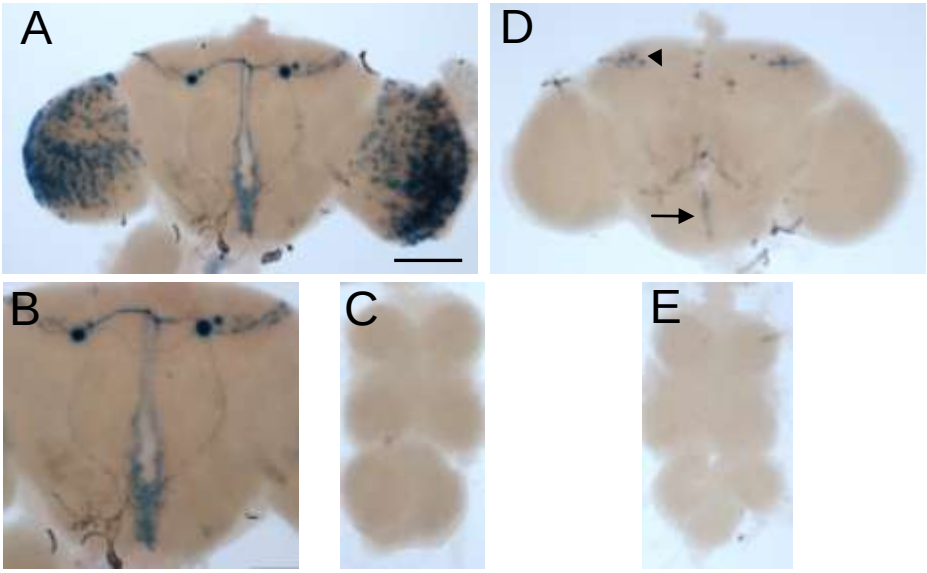


Fig. 2-12. ms-aCrz and pDLP *cis*-acting element between 241-bp and 290-bp upstream region. The reporter gene expression driven by 504Δ241-290 construct. (A-C) T3 line. X-gal signals are strongly detected in OL, DL-DLP and their neuronal projection (A and B), but not detected in VNC (C). (D and E) S2 line. Weak signals only in DL-DLP cells and projection in the dorso-lateral part (arrowhead) and esophagus region (arrow, D), but not detected in VNC (E). Scale bar indicates 100 µm.

Table 2-2. Larval expression driven by various Crz promoters

Length (bp)	Lines	Expression of the GFP or lacZ (each hemisphere)			Remarks
		DL	DM	VNC	
1185	T1	3	1	8	normal
	T2a	3	1	8	normal
802	S8	3	1	8	normal
	T1a	3	1	8	normal
	T5a	3	1	8	normal
504	T1a	3	1	8	normal
	T2a	3	1	8	normal
	T2b	3	1	8	normal
419	S2	3	0	8	No expression in DM cell of larvae and in medulla of adult
	S4b	3	0	8	No DM expression
	S5	3	0-1	8	ca. 50% specimens showed the gfp signal in DM
	S6	3	ND	8	ectopic cell expression
	T3a	3	0-1	8	ca. 25% specimens showed the gfp signal in DM
	T3b	3	0-1	8	ca. 50% specimens showed the gfp signal in DM
	T4	3	0-1	8	ca. 50% specimens showed the gfp signal in DM
321	X1a	3	0	4-5	Reduced number of cells in VNC
	X1b	3	0	4-6	Reduced number of cells in VNC
	S2b	3	0	2-4	Low expression in VNC
	S3a	3	0	0-1	Very low expression in VNC
	S3b	3	0	0	No expression in VNC
	S5a	3	0	0-2	Very low expression in VNC
	T2a	3	0	8	Normal in VNC
	T6	3	0	5	Reduced the number of cells in VNC
	T7	3	0	0	No expression in VNC
287	T1	2-3	0	0-3	Low expression in DL and VNC
	X2	0-2	0	0-3	Low expression in DL and VNC
	T3	2-3	0	1-3	Low expression in DL and VNC
	X4	3	0	2-3	very low expression in VNC
	S5	3	0	0-2	very low expression in VNC and ectopic expression in mushroom body
249	S2	0	0	0	No expression
	S4	0	0	0	No expression
	T1	0	0	0	No expression
504 Δ290-241	X1	3	1	3-4	Reduced number of cells in VNC, Weak expression in adult
	S2	3	1	3-4	Reduced number of cells in VNC, No OL expression and extremely weak DLP expression in adult
	S3	3	1	4-5	Reduced number of cells in VNC

Table 2-2 continued

	T3	3	1	3-4	Reduced number of cells in VNC
	T4a	3	1	4-6	Reduced number of cells in VNC
	T4b	3	1	4-5	Reduced number of cells in VNC
504 Δ249-311	T3	3	0	1-2	Weak expression in 2-3 cells in VNC
	T16	1	1	5-6	1 cell expression in DL with no projection
	T21	0-2	1	0-4	Weak expression in DL and VNC. No projection in DL
	T28	1-2	1	4-5	No projection in DL
	T38	1-2	1	2-4	Very weak projection in DL
	T39	3	1	4-6	Weak projection
504 ΔTATA	T2	3	1	6-8	1-2 cells are missing in VNC
	T3s	3	1	6-7	1-2 cells are missing in VNC
	T5w	3	1	6-7	1-2 cells are missing in VNC
380 Δ280-321	T3	3	0	8	Normal expression in DL and VNC
	T16	3	0	8	Normal expression in DL and VNC
	T21	3	0	8	Normal expression in DL and VNC
380 Δ241-279	X1,S2	3	0	0-1	Very weak expression in VNC. No projection in VNC
	T3	3	0	3-4	Very weak expression in VNC
	T4	3	0	3-4	Very weak expression in VNC
	T5	3	0	ND	Ectopic expression in VNC
	T6	3	0	0	Tv expression in VNC
	T8	3	0	2-3	Very weak expression in VNC. No projection in VNC
	T10	3	0	1-2	Very weak expression in VNC. No projection in VNC
380-241Δ280-321/w TATA	S4	0	0	0	No expression, ectopic expression in medulla
	T1	0	0	1-3	Weak expression in VNC
	T2	0	0	0-3	Very weak expression in VNC
380-241 Δ280-321 w/o TATA	S3, S7, T6	0	0	0	NO expression
	S4, S5, T9	0	0	0	Ectopic expression in brain, no specific expression
	T12w	0	0	1-2	Very weak expression in VNC

The number indicates the number of reporter gene expressing cell in each hemisphere.

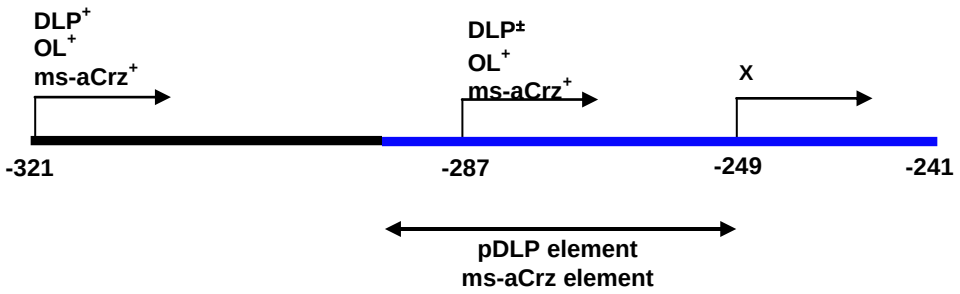


Fig.2-13. Schematic diagram of putative *cis*-acting elements for the transcriptional regulation of the *Crz* in the adult CNS. Negative numbers indicate nucleotide positions from the transcription start site**.**

Table2-3. Adult expression driven by various Crz promoters

Length (bp)	Lines	Expression of the GFP or lacZ (each hemisphere)			Remarks
		DLP	Medulla	ms-aCrz	
504	T1a	6-8	+++	N. T.	Normal
	T2a	6-8	+++	+	Normal
	T2b	6-8	+++	+	Normal
419	S2	6-8	—	+	No expression on medulla
	S5	6-8	+++	N. T.	
	S6	6-8	+++	N. T.	
	T3b	6-8	+++	+	Normal
321	X1a	N.T.	N.T.		
	S2b	6-8	+++		
	S5a	6-8	+++	+	Normal
	T2a	6-8	+++	+	Normal
287	T1	3-5	+++	+	Reduced number of DLP cells
	T3	1-5	—	+	Reduced number of DLP cells and no expression in medulla
	X4	1-5	+++		Reduced number of DLP cells
	S5	1-5	+++	+	Reduced number of DLP cells
249	S2	0	—		No expression
	S4	0	—	—	No expression
	T1	0	—	—	No expression
504 Δ290-241	X1	1-3	+	—	Reduced number of DLP and no expression in ms-aCrz
	S2	1-3	—	—	Reduced number of DLP and no expression in medulla and ms-aCrz
	T3	1-3	+++	—	Reduced number of DLP and no expression ms-aCrz
504 ΔTATA	S4s	6-8	+++	+	Normal
	S6w	6-8	++	+	Normal
	T5w	6-8	+	+	normal Crz expression but weak in medulla
380 Δ280-321	T3	6-8.	+++	+	normal Crz expression
	T16	6-8.	+++	+	normal Crz expression

N. T.: Not determined

screening, more than 18 randomly picked single plaques were amplified by PCR to evaluate the insert. From the 1st screening, the PCR products were highly heterogeneous (Fig. 2-16, upper panel), but after the 5th screening, the PCR products become more homogeneous, indicating enrichment of target proteins.

From the sequence analysis of these inserts, we obtained putative *Crz* regulator candidates; for examples, *protein on ecdyson puff, isoform A* (*PEP*, Fig 2-16, arrowhead on lower panel), *failed axon connection* (*fax*, Fig 2-16, asterisk on lower panel) and *CG12054* (zinc finger protein- C2H2 type). Further investigation will be required to verify their direct interaction with *cis*-element and regulatory function.

As an alternative approach, we performed affinity purification using putative Crz regulatory sequence as bait. After Coomassie staining, we found three putative *trans*-acting elements. One is strong and large protein and two weak and smaller proteins. The sizes of the protein are approximately between 60 to 80 KDa. Cytoplasmic protein extracts or no-bait controls did not show any specific bands (Fig. 2-17)

We also used web-based software (http://www.gene-regulation.com/pub/programs.html; AliBaba2.1) to identify putative binding factors. As a result, we found a sequence motif (CAGCAAATGC; e.g., Wentworth et al., 1991) within the -290::-241 interval that could be a binding site for a transcription factor *nautilus* (*nau*), a fly homolog of *MyoD* (Paterson et al., 1991). Crz-immunohistochemistry of the CNSs obtained from the *trans*-heterozygous *nau*-null (nau^{188}/nau^{armGFP}) mutants (Alcorta, 1991; Balagopalan et al., 2001; Wei et al., 2007), however, showed all endogenous Crz-immunoreactivity, suggesting that *nau* does not play a role as a *trans*-acting factor (Fig. 2-18 A). Interestingly, five out of six specimens

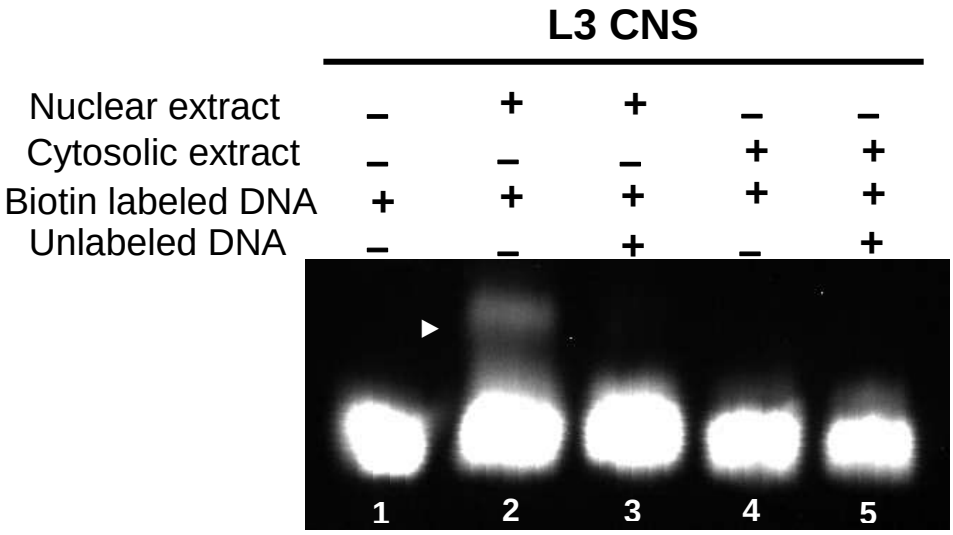


Fig. 2-14. Putative interaction between *cis*- and *trans*-acting elements. Electrophoretic mobility shift assay (EMSA) showed *Crz cis*-acting element interact with DNA binding proteins. A specific band shift is indicated by an arrowhead.

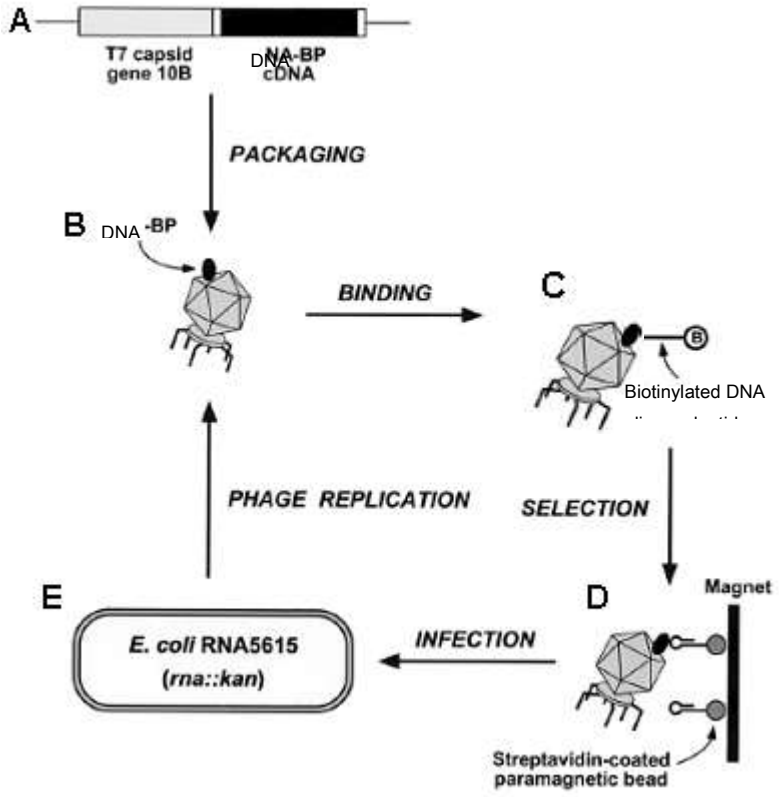


Fig. 2-15. Schemematic diagram of phage display. (A) The target cDNA library packed into T7 phage genomic DNA. (B) The target proteins display on phage capsid protein. (C) The biotynlated bait DNA binds to target proteins. (D) Immobilized DNA-protein complex using straptavidin-conjugated magnetic bead. (E) Immobilized phage is infeced to enrich the target. Modified from Danner and Belasco, 2001.

showed one to three weak Crz-immunoreactive neurons in close proximity to the DL neurons (arrow in Fig. 2-18 B). The cause of such ectopic expression is unknown. Visual inspection of the *Crz* regulatory sequence identified a consensus *Myocyte enhancer factor* 2 (*Mef2*) binding sequence (TTATTTTAAG) at -349::-340 (Andres et al., 1995). A very similar sequence motif was demonstrated to modulate transcription of a neuropeptide Prothracicotropic hormone (PTTH) in the silkworm, *Bombyx mori* (Shiomi et al., 2005). To test the possibility of *Drosophila Mef2* as a *Crz* regulator, Crz immunohistochemistry was performed in a hypomorphic *Mef2* mutant CNS ($Mef2^{30-5}/Mef2^{44-5}$), because homozygous *Mef2*-null alleles cause embryonic lethality (Lilly et al., 1995; Baker et al., 2005). Crz-immunoreactivity in this mutant CNS was normal, although two out of six specimens showed substantially weak Crz-immunoreactivity in a pair of vCrz neurons (arrow in Fig. 2-18 C). More definitive evidence for roles of *Mef2* as a *Crz* transcriptional regulator awaits viable *Mef2* null alleles.

Recently, basic helix loop helix (bHLH) transcription factor *DIMMED* (*DIMM*) was found to be expressed in the *Crz* neurons (Park et al., 2008). This result suggested a possibility for *Crz* regulation by DIMM. To test this possibility, Crz immunohistochemistry was performed in DIMM over-expression in *Crz* neuron by driving *Crz-gal4* driver. In early larval stage (1st and 2nd instar), Crz immunoreactivity was normal (Fig. 2-19 A), but after 3rd instar larva, DL neuron number increased from 3 to 6, and DM Crz immunoreactivity was enhanced (Fig. 2-19 B). Previously, we reported transient expression of *Crz* in developing pupae near the antennal lobe and in the subesophageal area (Lee et al., 2008). This expression was also enhanced significantly (Fig. 2-19 C, arrowhead). The molecular mechanisms underlying such

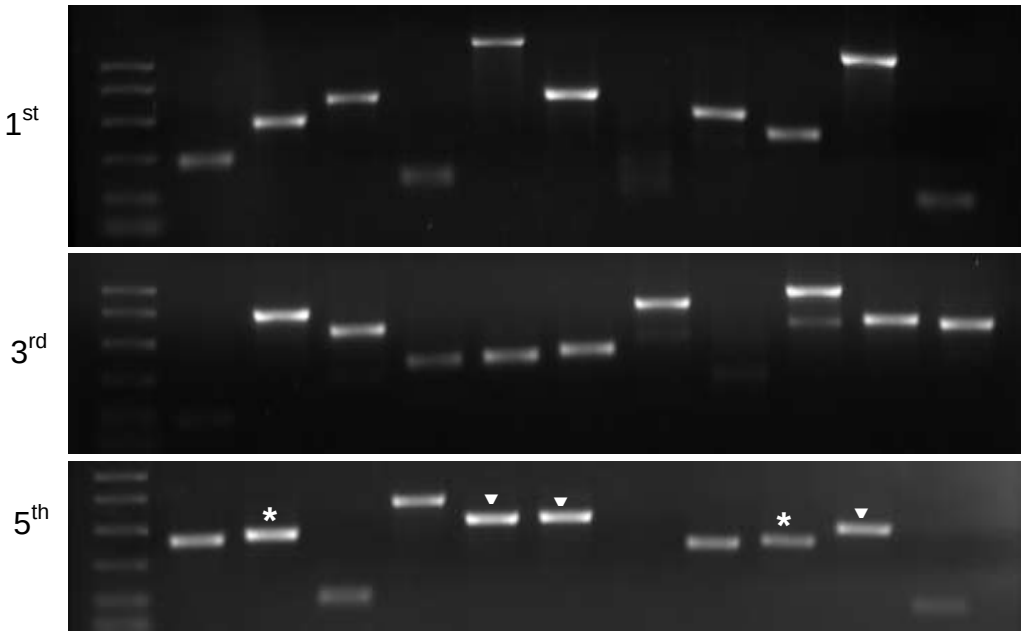


Fig. 2-16. Phage display to find *Crz* specific *trans*-acting element. After the 1st screening, the clones are highly heterologus. After the 5th screening, some clones contain identical PCR product, indicating a sign of enrichment (arrowhead and asterisk).

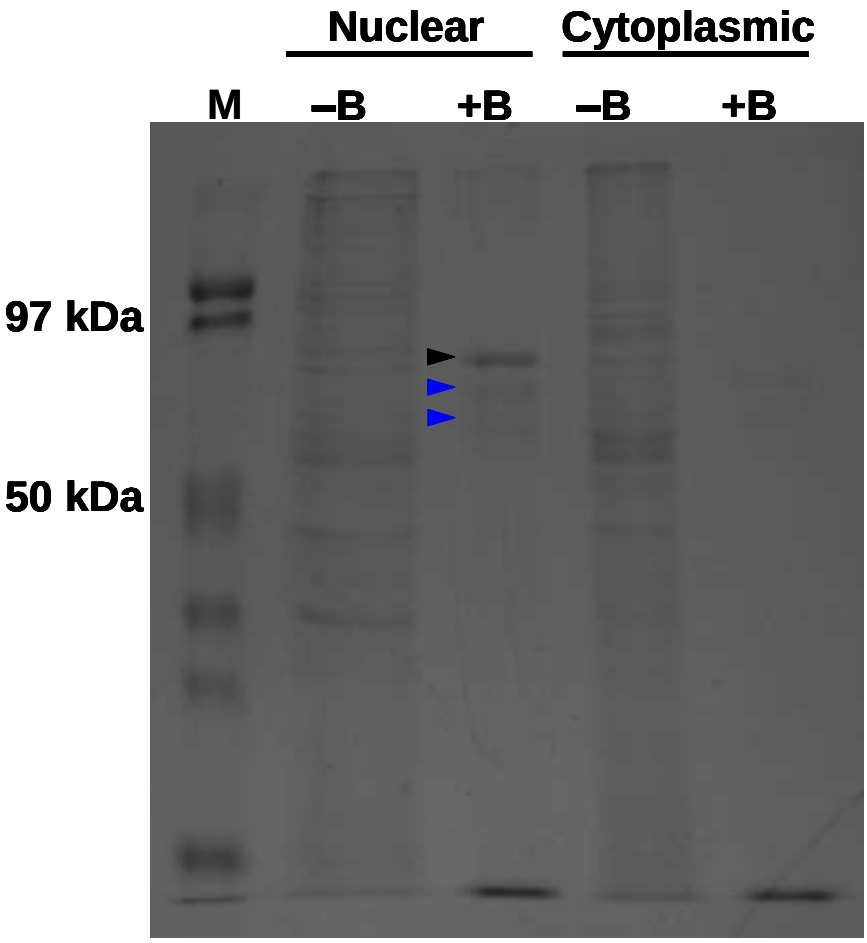


Fig. 2-17. Affinity purification for *Crz* specific *trans*-acting elements. After purification, three putative binding proteins are detected (lane 3). One is strong and large (black arrow head), and the other two are weak and smaller (blue arrow head). Without biotinylated DNA bates, lots of proteins are detected both nuclear and cytoplasmic protein extracts (Lane 2 and 4). With biotinylated DNA bait, only nuclear protein extracts show specific bands (Lane 3). M indicates protein size marker.

enhanced Crz immunosignals by DIMM over-expression are currently not understood.

Intriguingly, vCrz cells, which normally undergo programmed cell death within 6 hours after puparium formation, were detectable even in adult stage (Fig. 2-19 C and D). This result suggests that DIMM somehow plays a role as an anti-apoptotic factor. It is unknown how DIMM over-expression suppresses developmentally regulated *Crz* neuronal cell death and whether this is also true for other doomed tissues.

To increase the amount of DIMM protein in *Crz* neurons, we used two copies of the *UAS-dimm* recipient (*y w*; *UAS-dimm*^{2A3}; *UAS-dimm*^{3Ab}; courtesy by Dr. Dongkook Park at Washington University in St. Louis) and two copies of *Crz-gal4* driver (*Crz-gal4*^{X}; *Crz-gal4*^{S2b}). In contrast to one copy of DIMM, lots of optic lobe medulla cells (Fig. 2-20 A, B), one strong medial neuron (Fig. 2-20 C. arrowhead) and several ectopic neurons (Fig. 2-20 C. arrow) expressed *Crz* in each hemi brain. This result suggests that amount of DIMM protein is important for *Crz* translation in the optic lobe.

V. DISCUSSION

Like the genomes of many other multicellular eukaryotes, the majority of the euchromatic region of the *D. melanogaster* genome (~80%) does not code for polypeptides. One important role played by the non-coding sequences is to regulate spatial, developmental, gender-specific, temporal, and conditional transcription of genes Biggin and Tjian, 2001; Markstein and Levine, 2002). In this study, we attempted to define specific *cis*-regulatory regions that control *Drosophila Crz* transcription in three distinct neuronal groups in the larval and two distinct neuronal groups in adult CNS.

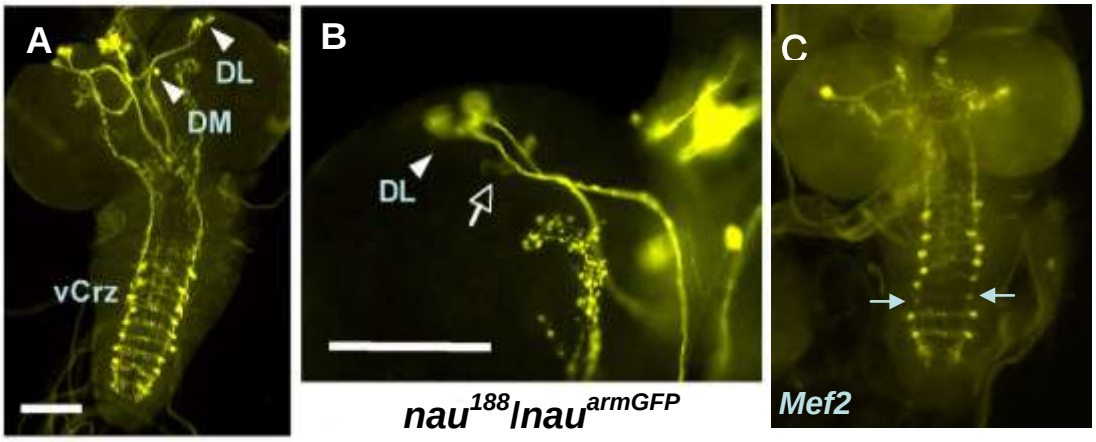


Fig. 2-18. *Mef*2 and *Nau* in *Crz* neurons (A) Intact Crz immunoreactivity in a *trans*-heterozygous nau-null mutant (nau^{188}/nau^{armGFP}). Similar normality was observed in homozygous nau^{armGFP} allele (not shown). (B) An image with higher magnification of Crz-immunoreactive neurons in the brain of nau^{188}/nau^{armGFP}. Weakly stained ectopic Crz-immunoreactive neurons are indicated by an arrow. (C) Crz immunoreactivity in the *trans*-heterozygous *Mef2* hypomorphic mutant CNS ($Mef2^{30\text{-}5}/Mef2^{44\text{-}5}$). vCrz neurons with noticeably weak signals are indicated by arrows. Scale bar indicates 100 µm

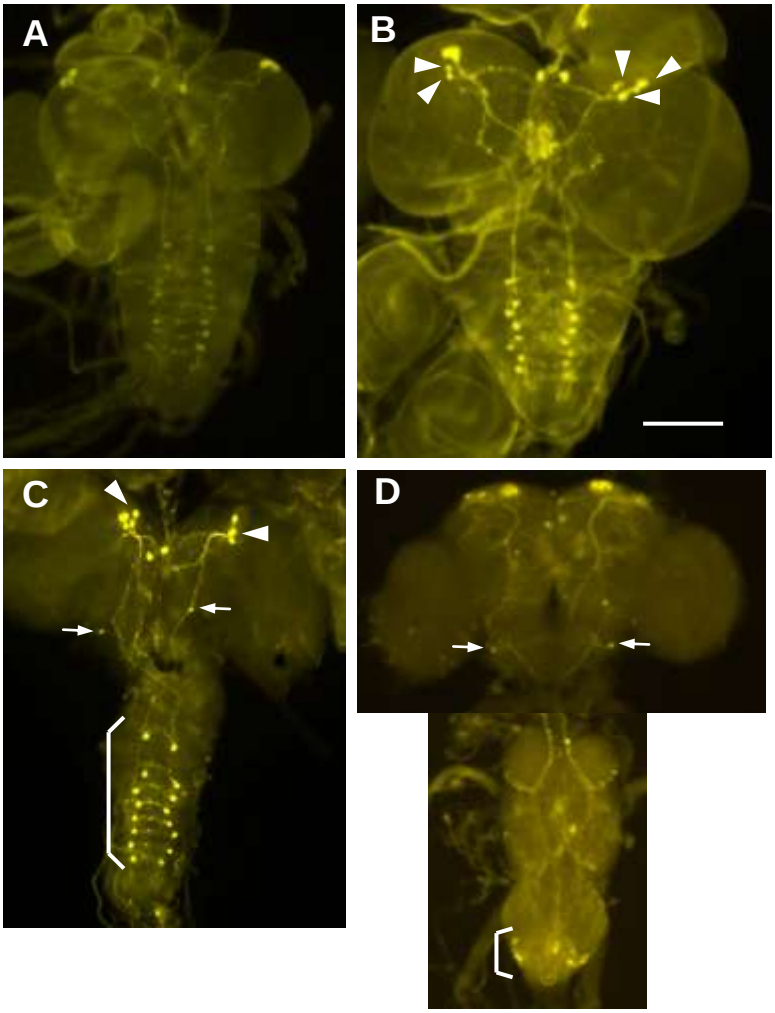


Fig. 2-19. DIMM overexpression enhanced of immunoreactivity and protected programmed vCrz cell death. (A) Early larval stage (younger than 2nd instar) did not show any ectopic Crz expressing cells. (B) Third instar larval stage. Three more *Crz* immunoreactive cells are shown each hemibrane (arrowhead). Immunoreactivity of DM cell is also enhanced. (C) One day old pupae shows extra DL (arrowhead), enhanced DM and one subsophageal cell (arrow) in each hemibrane. The vCrz cells are protected from programmed cell death (bracket). (D) In adult brain, transient subsophageal *Crz* neuron and vCrz are still remaining (white arrow and bracket). Scale bar indicates 100µm.

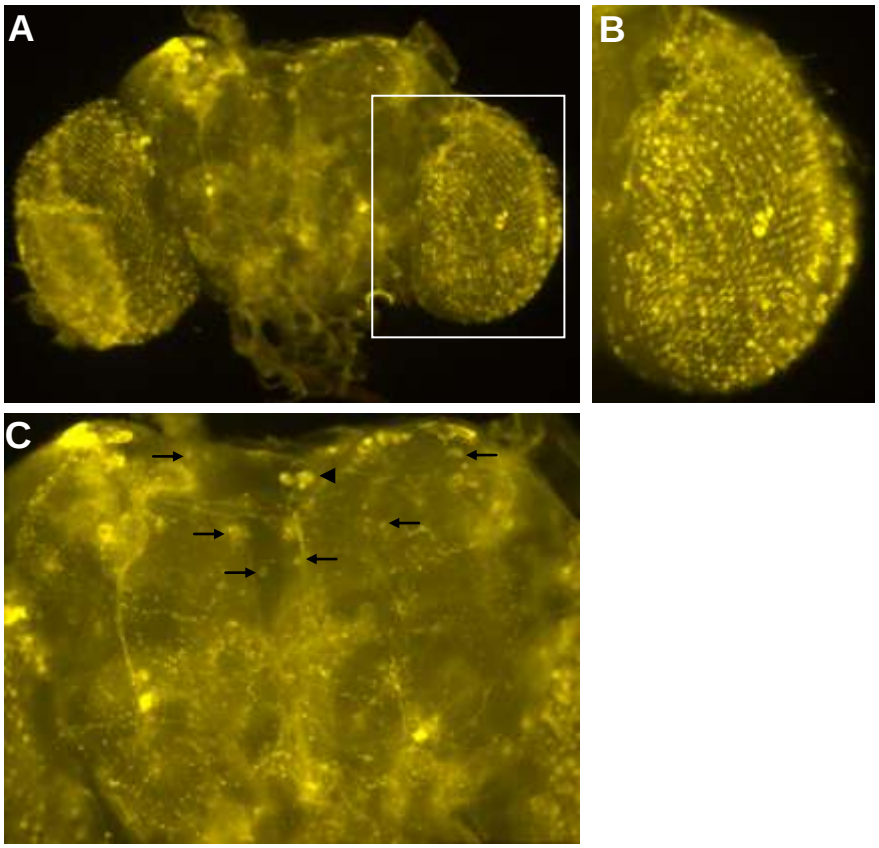


Fig. 2.20. Two copies of DIMM overexpression in the *Crz* neurons induced ectopic *Crz* expression in optic lobe medulla and brain region. (A) One or two days old adult brain showed strong expression of *Crz* in optic lobe (white rectangle). (B) High magnification in OL region. (C) High magnification of the brain. One pair of strong medial neuron (black arrowhead) and several ectopic *Crz* neurons (black arrow) are shown. Scale bars indicate 100µm.

cis*-acting elements for transcriptional regulation of the *Crz

Our promoter analyses suggest that multiple positively acting regulatory sites in the 5' upstream of the *Crz* gene are intricately involved in *Crz* expression in a group-specific manner. In larval stage, the region at -504::-419 is responsible for DM- specific expression, while an interval at -380::249 is allocated for expression in the DL and vCrz groups. Within the latter region, two separate regulatory elements, vCrz-d and vCrz-p, are found to be essential for vCrz specific expression. By comparison, three *cis*-regulatory sites (DL-d, DL-c, and DL-p) are important for DL expression and a combination of any two of them is required for normal DL expression. It is unlikely that sequences within intronic and 3'-UTR are involved in the regulation of *Crz* expression, because constructs lacking these regions recapitulated faithfully endogenous *Crz* expression. Our data also preclude a role for negatively acting *cis*-elements, as we did not observe any consistent ectopic expression mediated by promoter constructs tested here. By comparison to our results, expression of another *Drosophila* neuropeptide gene, *FMRFamide*, involves multiple positively or negatively acting *cis*-regulatory sequences, and these elements are distributed not only in the 5' upstream region but also in intronic sequences (Alcorta, 1991; Schneider et al., 1993; Benveniste et al., 1998; Benveniste and Taghert, 1999).

The TATA box does not play a significant role as a basal promoter element for *Crz* transcription in most *Crz* neurons. The TATA motif is the best-characterized core promoter for binding of basal transcription initiation complex (TFIID), and is essential for the transcription of many genes (e.g., Kutach and Kadonaga, 2000). However, there are few in vivo studies evaluating the role of the TATA box as a promoter of

neuropeptide genes. Surprisingly, the absence of TATA did not affect *Crz* promoter activity in most neurons, except for the posterior-most vCrz neurons (Fig. 2-6 H and I). This result suggests that other elements might be functionally redundant with the TATA-box. One such candidate is the cap-site consensus (or Initiator), which is able to direct accurate transcription initiation of TATA-less promoters (Smale, 1997). Interestingly, the Crz upstream sequence contains putative Initiator (TCA^{+1}GAC; +1 indicates transcription-start) (Fig. 2-5; cf. Arkhipova, 1995), raising the possibility that Initiator compensates for the loss of TATA. Thus, it would be interesting to determine activities of an upstream sequence lacking both TATA and Initiator. Nevertheless, that the TATA-box is an indispensable *cis*-element for the posterior-most vCrz neurons implies that fundamental mechanisms of the transcriptional initiation in these neurons are different from those in other *Crz* neurons.

By comparison with larval specific element, adult specific *cis*-acting element exists between -320-bp and -250-bp upstream region. Adult specific expression is subdivided into three different groups: DLP, ms-aCrz and medulla region. Interestingly, in medulla region, *Crz* transcripts are likely untranslated. These medulla specific transcripts were produced in an age-dependent manner, as older flies produced more mRNAs than younger flies, which suggested unknown age-related factors involved (Choi et al., 2005). From our results, we could not separate medulla specific *cis*-acting element, but we separated ms-aCrz specific *cis*-acting element. Without -290:: -249 region, construct could not drive reporter gene expression in ms-aCrz (Fig 2-11 and Fig. 2-13). To identify sex-specific trans-acting element, further analysis will be needed.

Putative *trans*-acting factors

Results from EMSA indicate the existence of specific *trans*-acting factors for *Crz*'s *cis*-elements; however, identification of such factors has been a challenge. To isolate, *Crz*'s *trans*-elements, we performed affinity purification using putative Crz regulatory site as bait. We got three strong candidates (Fig. 2-17), however, those candidates need to identify. To confirm whether DNA-binding protein, we will need to do further investigations such as Mass-Spectroscopy for protein identification and in vitro binding assay using purified proteins. Putative sequence-specific binding factors can be predicted by the identification of their cognate binding sequence in the regulatory region. For instance, *in silico* analysis eventually led to the identification of the *Mef2* as a regulator for PTTH expression in the brain of *Bombyx mori* (Shiomi et al., 2005). Similar attempts identified two interesting sequence motifs within the *Crz* regulatory region, which are putative binding sites for *Mef2* and *nau*. *Mef2* is a transcription factor containing conserved MADS domain, and *nau* is a member of the bHLH transcription factor family (Paterson et al., 1991). These two factors are important for embryonic and post-embryonic myogenesis (Balagopalan et al., 2001; Baker et al., 2005; Wei et al., 2007). Expression of *Mef2* was also found in the *Drosophila* brain, implicating its role in the nervous system (Schulz et al., 1996). Our genetic data, however, did not support *nau* and *Mef2* as *Crz* transcriptional regulators in most of endogenous *Crz* neurons. Since embryonic lethality of *Mef2*-null alleles does not permit us to assess Mef2's role conclusively, it is necessary to develop cell-specific knockouts of the *Mef2*, for instance, using RNA interference.

A bHLH transcription factor *DIMMED* (*DIMM*) is expressed in *Crz* neurons. DIMM is an important regulator for peptidergic neuronal development in *Drosophila* (Park et al., 2008). In wild type adult optic lobe medulla, *Crz* is transcribed, but not translated. Our DIMM overexpression data in *Crz* neurons suggest that DIMM controls *Crz* expression during development in a dose dependent manner. In wild type, one or two days old adult flies does not show any *Crz* transcripts in OL region, and OL specific transcripts never translated, but two copies of DIMM overexpression in Crz neurons, flies showed strong Crz proteins in OL region less than two days old. This result suggested that DIMM also accelerates development and changes cell fates. However, the molecular mechanisms are still remaining in mystery.

Comparative genomic analysis for finding *cis*-elements

Since the completion of genome projects targeting several *Drosophila* species (http://flybase.bio.indiana.edu/), alignment of non-coding sequences among genomes of these species has been used to identify conserved sequence motifs (e.g., Keightley and Johnson, 2004; Gauthier and Hewes, 2006; Halligan and Keightley, 2006) as it is generally assumed that such selectively constrained sequences might be of functional significance (Zhang and Gerstein, 2003). Here, we found four regions that are highly conserved among 5'-upstream sequences of the *Crz* genes identified from four *Drosophila* species. Expression analyses using several internally deleted upstream sequences revealed an interval at -279::-249 as an important *cis*-regulatory component for vCrz as well as DL expression (vCrz-p or DL-p element, Fig. 2-12). Although this region is nearly identical among the *Drosophila* species (Fig. 2-5), *504Crz*$^{\Delta 241\text{-}290}$*-gal*4

driver is sufficient to DL expression, but insufficient to vCrz expression like *321Crz-gal4* driver. This result addresses that another element needs to normal expression of vCrz. As previously mentioned, vCrz expression requires the cooperation of vCrz-d and vCrz-p elements (Fig. 2-12). Because of the latter vCrz-p element is absent in the*504Crz*$^{\Delta 241-290}$*-gal*4 driver, subnormal activity within vCrz neurons results from incomplete interactions between vCrz specific *cis*-acting elements and *trans*-acting factors.

Chapter Three

Heterologous Regulation of Crz Expression

I. ABSTRACT

During evolutionary process, gene regulatory mechanisms change and adapt in a species-specific manner. Although the functions of insect neuropeptide *Corazonin* are highly diverse, the basic expression pattern is conserved among insect orders. In *D. virilis*, Crz expresses in four pairs of brain neurons and eight pairs of ventral nerve cord in larval stage. In adult, number of brain Crz expressing neurons increased to 6-8 pairs in each hemi brain. Heterologous transgenic analysis showed *Crz cis*-regulatory elements of *D. virilis* are recognized by unknown adult abdominal ganglion specific transcription factors in *D. melanogaster,* resulting in ectopic expression of in host.

II. INTRODUCTION

Expression pattern of *Crz* in CNS of representative six insect orders was investigated using antiserum against corazonin: *Ctenolepisma lineate* (Zygentoma), *Locusta migratoria* (Orthoptera), *Oxya yezoensis* (Orthoptera), *Gryllus bimaculatatus* (Orthoptera), *Arge nigrinodosa* (Hymenoptera), *Pyrrhocoris apterus* (Hymenoptera), *Athalia rosae* (Hymenoptera), *Bombix mori* (Lepidoptera) and *Anomala cuprea* (Coleoptera). The Crz-like proteins were expressed in the brain and ventral nerve cord

in all tested insects except albino mutant of *L. migratoria* and the beatle *A. cuprea* (Roller et al., 2003).

In *Drosophila* species (*D. melanogaster, D. simulans, D. erecta* and *D. virilis*), *Crz* was cloned and expression patterns have been well investigated in larval and adult stage. Although sequence similarity showed only 62% between *D. melanogaster* and *D. virilis* in entire open reading frame, spatial expression pattern of *Crz* is highly conserved throughout evolution, as *Crz* neurons is restricted to three distinct groups of neurons in the larval CNS (Choi et al., 2005). In the adult stage, 6 to 8 of DLP neurons were detected in each hemisphere. Interestingly, two pairs of male specific abdominal ganglion cells (ms-aCrz) were found in *D. melanogaster* (Lee et al., 2008). These data suggest conserved regulatory mechanisms of *Crz*, despite its sequence divergence between the two *Drosophila* species.

In this report, we introduced *D. virilis Crz* upstream fragment into the *D. melanogaster* to understand the inter-specific regulation of *Crz*. *D. virilis* belongs to a different sub-genus, *Drosophila* and diverged from the *D. melanogaster* lineage about 60 million years ago (Tamura et al., 2004). Given such long evolutionary history, our heterologous promoter analysis is intended to understand co-evolution of *cis-trans* transcriptional regulators.

III. MATERIALS AND METHODS

Fly Strains

Flies were raised on a standard cornmeal-yeast-agar medium at room temperature. *Yellow white* (*y w*) or *white*^{1118} (*w*^{1118}) strains were used as a host strain

for germ line transformation and normal control. Transgenic *UAS-lacZ* and *UAS-mCD8GFP* reporter lines were used for the detection of *Crz* promoter activities. *D. virilis* flies were obtained from Tocson stock center.

Identification of 5' flanking sequences of *D. virilis Crz* gene

On the basis of the *Crz* gene sequence in *D. virilis* we previously reported (accession no. AJ851893, Choi et al., 2005), we performed an inverse PCR to obtain 5'-upstream regulatory sequence for the *Crz* gene in *D. virilis* according to the procedures described for *D. melanogaster* (www.fruitfly.org/about/methods/inverse.pcr.html). Thus, genomic DNA was first purified from 15 flies using DNAsol solution as previously described. The purified genomic DNA was resuspended in 100μl 10mM Tri-HCl (pH 8.5) and kept on -20℃ before using. Eight μl of purified *D. virilis* genomic DNA was digested with *Msp* I (+840) or *Taq* I (+873) in 40μl at 37℃ for 2 hours. After heat inactivation at 65℃ for 20 min, half of the digested DNA was ligated with T4 DNA ligase in 400μl at 4℃ for overnight. After ethanol precipitation, the ligated DNA was resuspended in 20μl elution buffer. Two μl of resuspended genomic DNA was used for PCR (Fig. 3-1). The following primer was used for forward primer 5'-CCTGTTGCAACTGCAGCACT for *Msp* I digestion and *Taq* I digestion (from +752 to +771). The following primer was used for reverse primer: 5'-TTCACACTTGCCAAGAATCCA for *Msp* I digestion (From +47 to +67) 5'-TCATGCGTGGCCAAGATGGCAT for *Taq* I digestion (From -243 to -222). To get 1.2-

kb upstream region, we searched for *D. virilis* genome database. This resulted in one sequence (scaffold_53:348198-348777) that is highly homologous to the Crz encoding sequence. To generate *DvCrz-gal4* constructs, 434-bp upstream and 1.2-kb upstream region of *D. virilis Crz* were amplified by PCR using *D. virilis* genomic DNA as a template. The following primers were used as forward; 5'-GGAGATCTTCGATTTGGCCATTTGCAATGCG for 434-bp upstream, 5'-GGAGATCTGTGCAAAGTGCAAATG for 1.2kbp-upstream, and a common reverse primer; (5'-TGGCCAAACCACACACAAGCAG). For directional cloning forward primers added *Bgl* II site (underline). The PCR products were subcloned into pGEM-T easy vector and performed sequencing reaction. The *DvCrz* upstream fragments were isolated from *Bgl* II/ *Eco*RI restriction enzyme digestion and subcloned into *pPTGAL* fly transformation vector. The constructs were introduced into *y w* or w^{1118} host by conventional *Drosophila* transformation.

Expression Analysis

See Chapter 2 materials and methods

Results

***D. virilis Crz* upstream fragment**

To gain *D. virilis Crz* (hereafter *DvCrz*) upstream sequence, we performed inverse PCR. Based on the *D. virilis Crz* gene sequence (Choi et al., 2005), we designed inverse PCR primers and amplified upstream fragment. From the first trial

with *Msp* I digestion, we obtained 274-bp upstream fragment. To get further upstream region, we did another round of inverse PCR using *Taq* I restriction enzyme digestion. As a result, 434-bp upstream fragment was obtained: this fragment was confirmed by PCR amplification of *D. virilis* genomic DNA. Furthermore, this sequence matches perfectly to one that later published in the flybase. This region has relatively low similarity compared to other species; for example, *DvCrz* has ~40.2% sequence identity with *DmCrz* upstream region, but *D. simulans*, which is including in same sub-genus with *D. melanogaster*, has 92% and *D. pseudoobscura*, which is including same sub-genus with *D. virilis*, has 58.3% similarity (Fig. 2-5). To investigate more upstream region of *DvCrz*, we searched *D. virilis* genome database using corresponding to putative Crz peptide sequence. We found one sequence (scaffold_53:348198-348777) that is highly homologous to the CRZ encoding sequence. Using sequence available in the database, we amplified 1.2-kb upstream construct (1.2-kb *DvCrz*; Fig. 3-1).

Heterologous promoter analysis

Spatial *Crz* expression patterns in the larval CNS of *D. melanogaster* are comparable to those of *D*. *virilis* (Choi et al., 2005). We hypothesize that such consistent *Crz* expression patterns between the two distantly related species are the result of a conserved mode of interaction between cis- and trans-acting factor(s). If this is the case, then the 5' upstream sequence of the *DvCrz* gene would likely direct reporter expression in endogenous *Crz* neurons when introduced into the *D. melanogaster* genome. To test this hypothesis, we generated DvCrz-gal4 transgenic

lines using the 434-bp upstream sequence of the *DvCrz* (Fig. 3-1). In the larval CNSs, the *DvCrz-gal4* transgene induced GFP expression in the DL- and vCrz-like neurons,

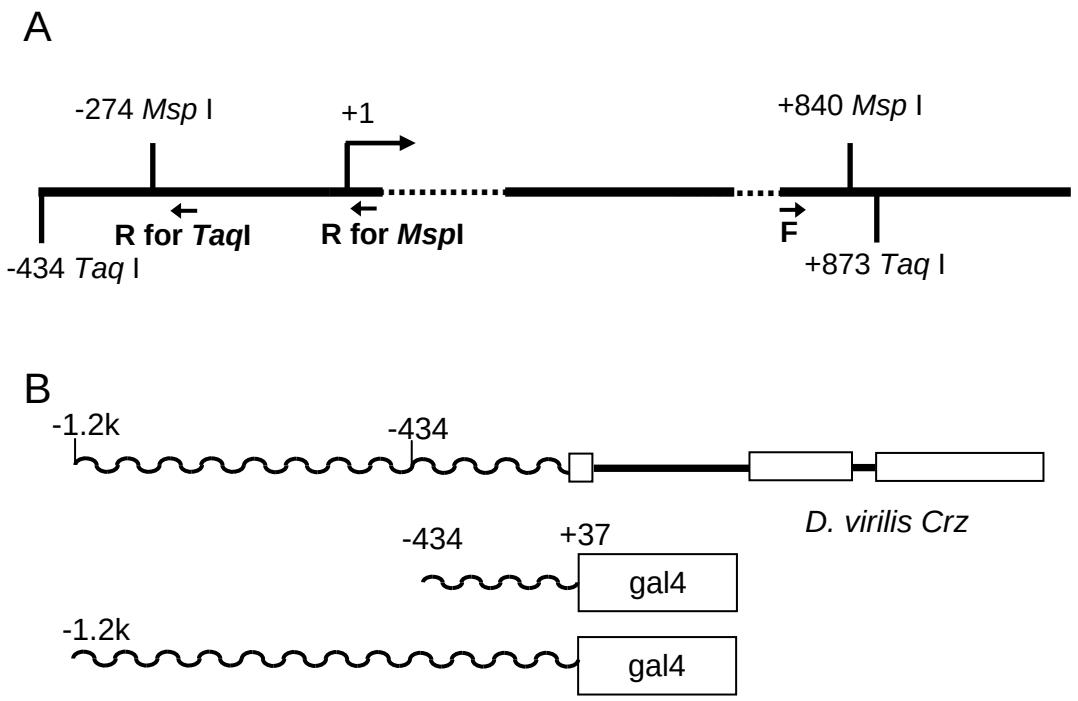


Fig. 3-1. Diagram of *D. virilis Crz* genomic DNA structure and gal4 fusion constructs. (A) *D. virilis* Crz genomic DNA structure. Numbers indicate nucleotide positions from the transcription start site. Dashed lines indicate introns in *D. virilis Crz* gene. R stands for reverse primer, and F is a forward primer for inverse PCR. (B) *DvCrz-gal4* constructs. Wavy lines designate 5' upstream region (-434 to +37 and -1.2 k to +37) of the *DvCrz* gene fused to the gal4 coding sequence.

but none in the DM-like neurons in all three lines tested (Fig. 3-2). To confirm whether these neurons are genuine *Crz* neurons, each *DvCrz-gal4* line was crossed to a *UAS-CD8GFP* line, and then larval CNSs of the progeny were processed for Crz-immunohistochemistry. In this type of double-labeling experiment, GFP signals represent *DvCrz* promoter activities, while anti-Crz marks endogenous *Crz* expression sites. In the protocerebrum, both signals were clearly superimposed in all of the DL neurons, but only Crz-immunoreactivity was evident in the DM neurons (Fig. 3-2 C, F, and I). In the VNC, GFP expression varied among the lines tested; while most of the vCrz expression (except in one or two neurons) was observed in S4 line (Fig. 3-2 A), the expression was apparent only in ca. 50% of the vCrz neuron in T13 or T14 drivers (Fig. 3-2 D and G). Consistent with the latter results, T13 mediated expression of a proapoptotic gene, *reaper*, spared Crz-immunoreactive DM neurons and 7-8 vCrz neurons (arrowheads in Fig. 3-3).

The lack of DM expression by the 434-bp *DvCrz* promoter could be accounted for by the exclusion of *cis*-acting regulatory site(s) required for DM expression from this sequence, as was observed for the 419-bp upstream sequence of the *D. melanogaster Crz* (Fig. 2-3). Nevertheless, promoter activities of the 434-bp *DvCrz* upstream fragment within the DL and vCrz groups imply that *trans*-acting factors responsible for *Crz* transcription within these two neuronal groups are able to recognize *DvCrz* transgenic promoter. However, such heterologous molecular interactions are likely to be suboptimal in the vCrz neurons, as reflected by subnormal reporter activities in the vCrz neuronal group.

In contrast 434-bp *DvCrz* construct, 1.2-kb *DvCrz* produced DM expression in some lines (arrows in Fig 3-4 A, B and D). The vCrz expression was also variable. For example, T4 line produced 8 pairs of reporter gene expression in vCrz (Fig 3-4 B), while S8 line produced 7 pairs of reporter gene expression (Fig 3-4 A) and the last pair was missing. The other lines were missing 2-3 pairs of vCrz expression (T1, T2 and T3, arrowheads in Fig 3-4 C-E).

In adult, 434-bp *DvCrz* and 1.2-kb *DvCrz* did not show any differences. Reporter gene expression was observed in DLP and abdominal ganglion (hereafter abCrz): Quite interestingly however, neither upstream fragments did drive expression in the optic lobe medulla (Fig 3-5: cf. Fig 2-10, 11 and 12). This is consistent with the lack of OL expression in D. virilis (Choi et al., 2005).

Interestingly, we found numbers of reporter gene expression in the abdominal ganglion driven by *DvCrz-gal4*. Two subgroups were detected: 5-6 pairs of strong abdominal Crz and 2-4 pairs of weak and fuzzy abdominal Crz (Fig 3-6, arrowhead). Since these neurons are normally *Crz*-negative, it is likely that promiscuous interaction between host factors and *DvCrz cis*-elements generates such ectopic reporter expression.

Discussion

In this study, we tested neuropeptide regulation using heterologous promoter analysis. Previously, the *Crz* expression pattern is almost identical among *Drosophila* species (Choi et al., 2005). This result suggested that the basic *Crz* regulation machinery is conserved even distantly relative species. To test this hypothesis,.

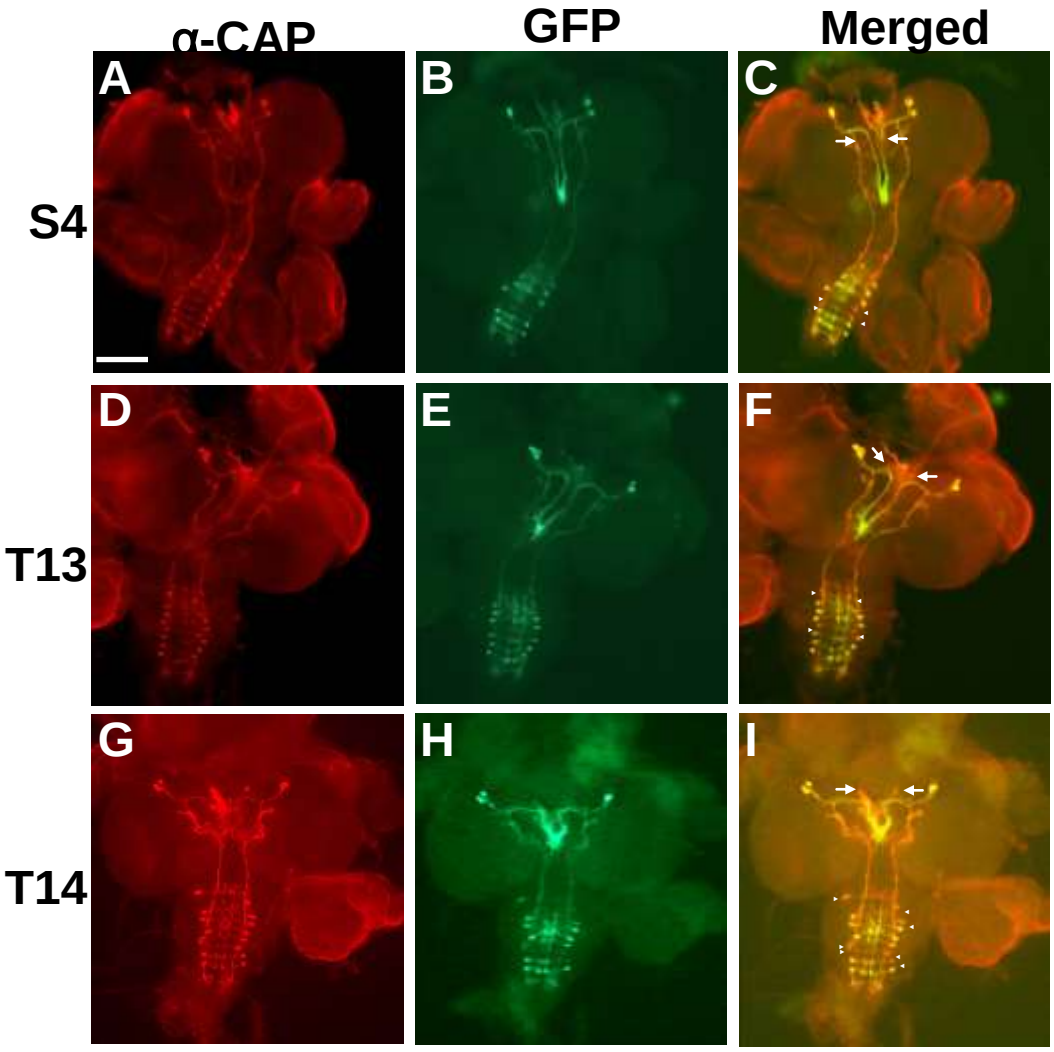


Fig. 3-2. *D. virilis* 434 bp upstream Crz promoter (Dv434Crz-gal4) activity in *D. melamogaster*. (A, D, and G): Expression of Crz detected by anti-CAP activity in wandering larval stage. (B, E and H): Expression of mCD8GFP driven by Dv-Crz-gal4 drivers. (C, F and I): Merged image between immunoreactive and reporter gene expression. Dv434Crz-gal4 promoter is unable to drive reporter gene in DM neurons (arrow). Scale bar indicates 100µm.

Fig. 3-3. Crz-immunoreactive neurons in the progeny from *DvCrz-gal4*^{T13} X *UAS-reaper* cross. Proapoptotic protein Reaper expression driven by *DvCrz-gal4*^{T13} induces cell death in all DL and about 50% of vCrz. Surviving Crz-immunoreactive cells in DM and vCrz are indicated by black arrow head. Scale bar indicates 100 µm.

expression analyses using several internally deleted upstream sequences in *D melanogester* revealed an interval at -279::-249 as an important *cis*-regulatory component for vCrz as well as DL expression (vCrz-p or DL-p element, Fig. 2-5). Although the sequence of this region is nearly identical between *D. virilis* and *D. melanogaster*, the 434-bp *DvCrz* promoter construct directed reporter expression sub-normally in the vCrz neurons. As mentioned earlier, vCrz expression requires cooperative action of two separate regulatory sites, vCrz-d and vCrz-p (Fig. 2-12). Since the latter vCrz-p element is present in the 434-bp *DvCrz* promoter, subnormal activity of the *DvCrz* promoter within the vCrz neurons likely results from the incompatible vCrz-d element due to sequence divergence (Fig. 2-5). If the 434-bp sequence is adequate for vCrz expression in the native host (i.e., *D. virilis*), then insufficient vCrz expression driven by the 434-bp *DvCrz-gal4* transgene in *D. melanogaster* host can be interpreted by suboptimal interaction between the vCrz-d-like element in the *DvCrz* promoter and *D. melanogaster*'s transacting factor. Such inter-specific incompatibility might be an example of the co-evolution of *cis-trans* regulatory components in which changes in *cis*-element sequence are accompanied by compensatory mutations in the *trans*-acting factors in order to maintain functional compatibility within the same species Shaw et al., 2002; Wittkopp et al., 2006). More definitive evidence for this hypothesis should come from the comparison of in vivo activity of the *cis*-elements in the native host to that in alien species. In contrast to 434-bp *DvCrz-gal4*, 1.2-kb upstream fragment produced DM and vCrz neurons in *D. melanogaster* although showed variable results (Fig. 3-4). This variability might be come from the genomic environment of transposable element insertion site.

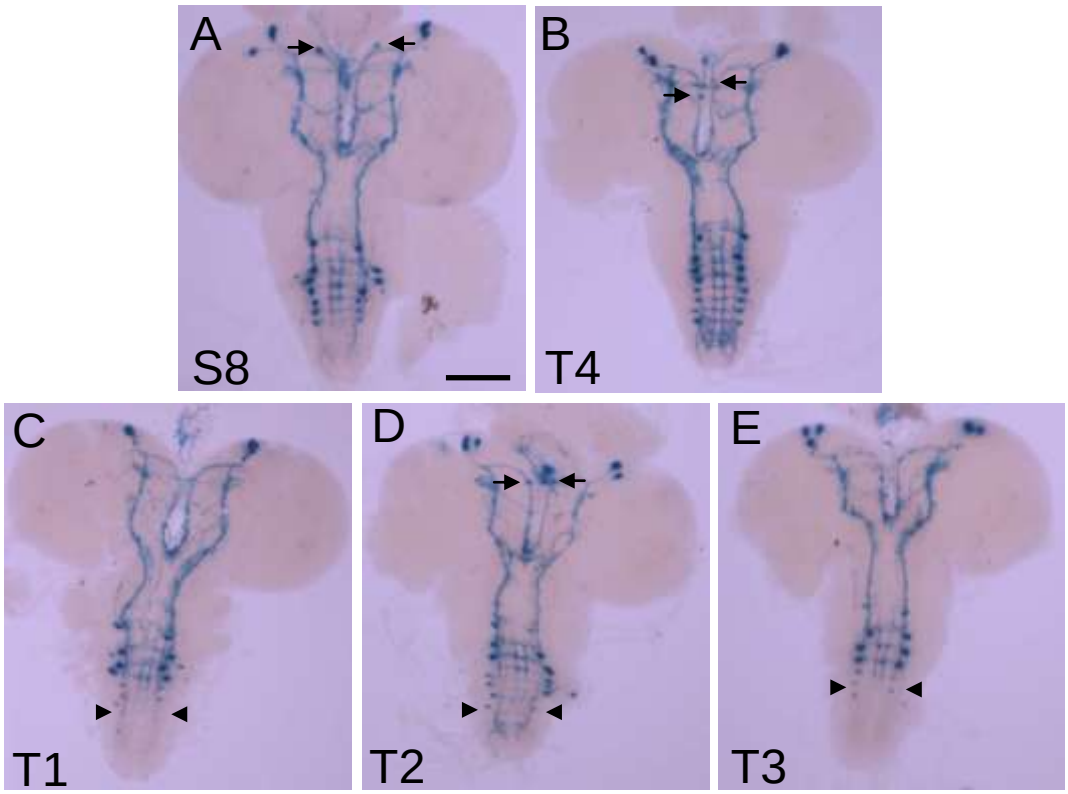


Fig. 3-4. *D. virilis* 1.2k upstream Crz promoter (Dv1.2kCrz-gal4) activity in *D. melamogaster*. (A, B) Normal reporter gene expressions are detected in DL, DM and VNC in S8 and T4 lines. (C-E), T1, T2 and T3 lines derive lacZ expression subnormally in the VNC region (arrowhead). Scale bar indicates 100µm.

In adult stage, 434-bp DvCrz driver is sufficient to produce adult specific Crz expression (Fig 3-5). For adult specific expression, *DmCrz* needs only 321-bp upstream fragment (Fig 2-9 A-C). The 434-bp upstream of *DvCrz* is comparable to *DmCrz*'s 419-bp upstream region, which is sufficient for adult expression (Fig 2-5). In *D. melanogaster*, *cis*-acting element for male specific abdominal *Crz* expression locates between 241-bp to -290-bp upstream region, therefore 434-bp *DvCrz* driver may produce reporter gene in ms-aCrz expression. Interestingly, *DvCrz* driver induce ectopic expression in abdominal ganglion and can be subgrouped strong and weak abdominal Crz (Fig. 3-6). It is perhaps because *DvCrz* promoter is recognized by unknown abdominal specific transcription factors in *D. melanogaster*. This result strongly indicates inter-specific gene regulation mechanism is changed *cis-trans* regulatory components during evolution.

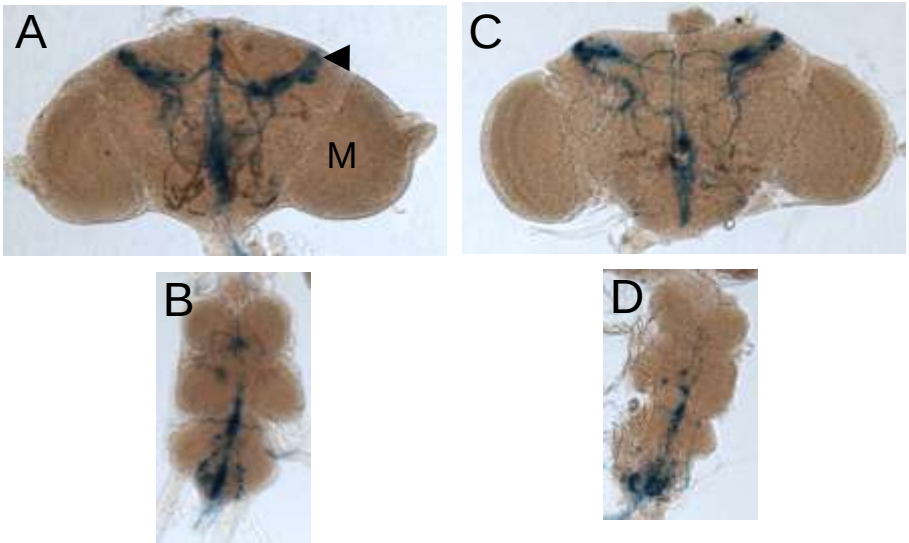


Fig. 3-5. Reporter gene expression by 434*DvCrz-gal4* showed on DLP (arrowhead), but not on optic lobe medulla. Two different representative lines (S4: A and B and T13: C and D) are shown. Scale bar indicates 100 µm. (M: optic lobe medulla)

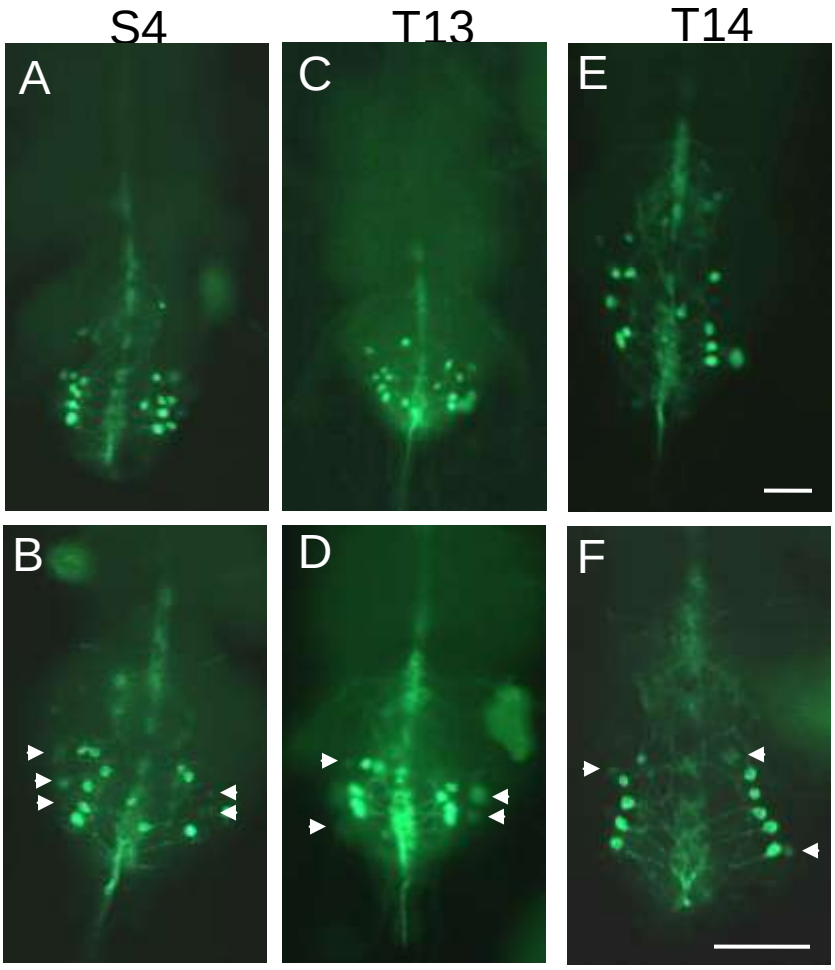


Fig. 3-6. *434DvCrz Crz* promoter is recognized by *D. melanogaster* in adult abdominal ganglion. Two groups of abCrz exist. Weak group of cells locates peripherally (with arrowhead). Upper panel shows different DvCrz-gal4 lines. Lower panel showed higher magnification of upper panel. Scale bar indicates 100µm.

Chapter Four

I Want to Escape from Hangover: *Corazonin* in Ethanol Metabolism

I. Abstract

Ethanol is an important environmental attractant for *Drosophila* species, serving as an energy source. Like mammals, low dose of ethanol causes euphoria, but high dose induces toxic effects. In locust, CRZ injection affected antennal sensilla development, which is important for chemical sensing. To identify the roles of *Crz* in *Drosophila*, we tested ethanol-related behavior in Crz-CD and CrzR-KD flies. Crz cell deficient (Crz-CD) flies and Crz receptor knock down (CrzR-KD) flies showed significantly delayed recovery from ethanol-induced sedation compared to control flies. This phenotype was ethanol-specific, indicating *Drosophila Crz* is involved in ethanol-related responses. Crz-CD and CrzR-KD flies did not affect ethanol absorption and degradation. Further analyses suggest that Crz-CD and *CrzR* mutant did not affect aldehyde dehydorgenase (ALDH) at transcription level, but reduced ALDH enzyme activity.

II. Introduction

Many *Drosophila* species breed in fermenting fruit, where ethanol can reach concentrations of several percent or higher (McKenzie and McKechnie, 1978; Gibson and Oakeshott, 1981). Thus flies have evolved to be tolerable to ethanol. The ethanol

resistance is one of the important selective marker to classify *Drosophila* species (Mercot, 1994). The model species *D. melanogaster* is one of the most ethanol resistant *Drosophila*, and more attracted to ethanol than other *Drosophila* species (David and Bocquet, 1975; Parsons and King, 1977). The ADH, which catalyses the oxidation of ethanol to acetaldehyde, is essential for ethanol degradation and resistance. *Adh*-null flies are extremely sensitive to ethanol (Bijlsma-Meeles and Bijlsma, 1988; Oudman et al., 1991), and ADH activity is positively correlated with ethanol resistance among *Drosophila* species (Mercot, 1994). In addition, within *D. melanogaster*, strains with higher ADH activity (*Fast* electoromorph) show higher ethanol resistance than less active *Slow* electoromorph (reviewed in Heinstra, 1993).

Another important enzyme in ethanol metabolism is ALDH, which catalyses the irreversible NAD^+-dependent oxidation of acetaldehyde to acetic acid. ALDH is considered the main enzyme responsible for the oxidation of acetaldehyde in mammals (Tank and Weiner, 1979). In humans, an inherited deficiency in the mitochondrial ALDH isozyme, ALDH2, causes a syndrome known as acute alcohol sensitivity, which is more prevalent in the Eastern Asian population (Impraim et al., 1982; Yoshida et al., 1984; Alcorta, 1991; Peng et al., 1999). After ingesting small amounts of ethanol, these people, experience a variety of unpleasant symptom caused by the accumulation of acetaldehyde, which is considerably more toxic than ethanol.

While it is clear that *Drosophila* has an active ALDH (Lietaert et al., 1985; Garcin et al., 1986; Heinstra et al., 1989; Anderson and Barnett, 1991; Heinstra, 1993; Leal and Barbancho, 1993), some studies suggested that ALDH is less important for ethanol metabolism in *Drosophila* than mammals (Geer et al., 1985; Heinstra et al., 1989; Geer

et al., 1993), because *Drosophila* ADH has capacity of oxidizing acetaldehyde to acetate by itself (Brooks et al., 1985; Eisses et al., 1985; Eisses et al., 1985; Geer et al., 1985; Heinstra et al., 1986; Heinstra et al., 1989; Alcorta, 1991). Unlike mammalian ADH, *Drosophila* ADH has quite different enzyme structure and evolutionally process, and also has weak ability to oxidize aldehyde (Hinson and Neal, 1972).

Like mammals, *D. melanogester* shows quite similar responses to ethanol with respect to the behavioral patterns (Bellen, 1998; Heberlein, 2000; Scholz et al., 2000; Wolf and Heberlein, 2003). At low doses, flies increase locomotion, but at high doses, fly movement is gradually decreased and finally intoxicated or sedated. In addition, flies acquired tolerance after a single dose treatment according to dose dependent manner, which is arbitrated by adaptation of brain activities (Scholz et al., 2000). Recurring ethanol exposure also induced chasing other male to try courtship behavior in male flies (Lee et al., 2008). These similar responses to ethanol between mammals and flies strongly suggested that ethanol affects fly and mammalian nervous system in a similar manner.

Fly neuropeptide F (NPF), a mammalian neuropeptide Y (NPY) homolog, involves in ethanol-related behavior. Deficiency in NPF/ NPF receptor (NPFR) showed decreasing alcohol sensitivity, but overexpression of NPF/NPFR increases ethanol sensitivity (Wen et al., 2005). Another fly neuropeptide *ammesiac* (*amn*) also participates in ethanol sensitivity. The *amn* mutant shows malfunction of memory (Quinn et al., 1979) and hypersensitivity to ethanol (Moore et al., 1998). The product of *amn* gene, AMN, stimulates cAMP synthesis and activates protein kinase A (PKA; Fig. 4-1, Bellen, 1998; Moore et al., 1998).

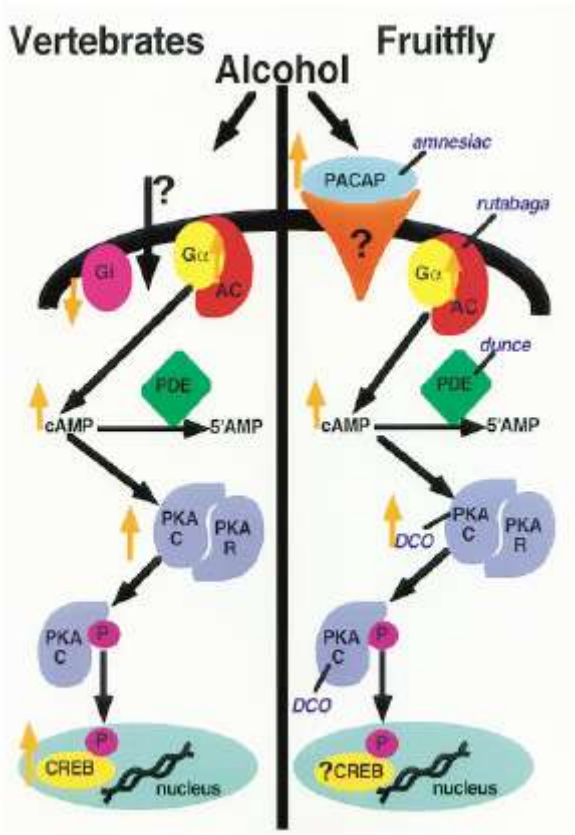


Fig. 4-1. Conserved molecular mechanism of cAMP pathway in acute and chronic ethanol exposure. A comparison of molecular components between vertebrate cultured neuronal cells (left) and alcohol sensitivity in *Drosophila* (right). Acute alcohol exposure causes up-regulation of the cAMP signaling pathway. Figure from Bellen, 1998. (PACAP, Pituitary adenylate cyclase activating peptide; AC, adenylate cyclase; PDE, phosphodiesterase; PKA, Protein kinase A; CREB, cAMP response element binding)

Recently, production of *Drosophila* Homer protein, in the ellipsoid body, a homolog of mammalian Homer 1 protein, is required for normal ethanol sensitivity and tolerance, as the Homer mutant flies increased the ethanol sensitivity and failed to develop tolerance after ethanol exposure (Urizar et al., 2007).

Insect neuropeptide, *Corazonin*, functions were investigated in diverse insect species, but universal roles were not identified yet. In this report, we investigated ethanol-associated function of *Crz* in *Drosophila*.

III. Materials and Methods

Crz RNAi constructs

Using wild type (Canton S) genomic DNA as template, primers PCZ5F (5´-GGTCAGAGCATATTAAAAACAGTTGTAAC) and CZintrnR (with a *Bgl* II site, 5´-ATAGATCTGCGATAGACAGCTGGAGGAA) were used to amplify the 'Crz promoter-exon1-intron1-partial exon2' region. The result product was named 'PCZ-EI'. The first exon of *Crz* gene was amplified using primers CZex1F (with a *Not* I site, 5´-TAGCGGCCGCAGACGCAGTTGTGATTCGAA) and CZex1R (with a *Bgl* II site, 5´-ATAGATCTCTCGAGCCTACGATCGCTGC), which was called 'CZ-E'. The 'PCZ-EI' fragment was cloned into pGEM-T easy vector; a clone (named pG-PCZ-EI) was selected in which the 5´ end of the insert was next to the T7 promoter sequence. The 'CZ-E' fragment was cloned into pGEM-T easy vector, called pG-CZ-E, in which the 5´ end of the insert was next to the Sp6 promoter sequence. Subsequently, *Bgl* II and *Pst* I were used to digest the pG-PCZ-EI vector, and the 300bp 'CZ-E' insert was removed

Table 4-1. Summary of the crossing for behavior assay

♀♀	♂♂
y w	*Crz-gal4^{T2a}*
y w	*UAS-hid/ CyO, y^{+}*
y w	*UAS-dronc*
y w	*Crz-gal4^{T2a}; Akh-gal4*
UAS-hid/ CyO, y^{+}	*Crz-gal4^{T2a}*
UAS-dronc	*Crz-gal4^{T2a}*
UAS-hid/ CyO, y^{+}	*Crz-gal4^{T2a}; Akh-gal4*
y w	*y w;; act-gal4/TM6c, Tb, Sb*
y w	*y w;; UAS-CrzR^{RNAiS3S}*
y w	*y w;; UAS-CrzR^{RNAiT17}/TM6c, Tb, Sb*
y w;; UAS-CrzR^{RNAiS3S}	*act-gal4/TM6c, Tb, Sb*
y w;; UAS-CrzR^{RNAiT17}/TM6c, Tb, Sb	*act-gal4/TM6c, Tb, Sb*
CrzR^{minos}	

from pG-CZ-E vector using the *Bgl* II and *Pst* I restriction enzyme. This CZ-E fragment was then ligated with *Bgl* II/ *Pst* I-digested pG-PCZ-EI vector to produce pG-PCZ-EIE vector that had the 1.4 kb Crz inverted repeat (ir) construct (Fig 4-7)

Since there is a *Spe* I site on the 500bp5´-upstream region, the *Spe* I and *Not* I were used to remove the Crz ir construct from pG-PCZ-EIE. The same enzymes were used to digest the *Drosophila* transformation vector, *pCaspeR4*, which was further treated with Shrimp Alkaline Phosphatase (SAP) to remove phosphate group for lower the background in transformation, and subsequently ligated with the 1.4 kb Crz-ir to generate *pCasp-CZ-ir* vector.

***Crz* Receptor Knockdown**

To generate *Crz receptor* RNAi construct under the *UAS* promoter, the -59 to +328 fragment of *CrzR* (+1 indicates the translation start site) was amplified by PCR from a previously cloned *CrzR* cDNA (pcDNA-CrzR-3.1; Courtesy by Dr. Young-Joon Kim from UC Riverside) template. Primers for PCR (forward, 5′-GGAGATCTCCCTAAACCACCGTCCAAACC: reverse, 5′-CGGAATTCATCCATCACGTGCTCCGGAAC) were used. The PCR product was digested with *Bgl* II/ *Eco*RI, purified, and then subcloned into the *sympUAS Drosophila* transformation vector (Giordano et al., 2002). The recombinant vector (*UAS-CrzR*^{RNAi}) was introduced into w^{1118} host via conventional germ-line transformation.

To ascertain knockdown of the *CrzR* expression, female *UAS-CrzR*^{RNAi} were crossed to universal driver *act-gal4*, and then the *CrzR* mRNA levels in the progeny were measured by RT-PCR. Briefly, total RNA from about 20 adult flies was purified by

TRIzol reagent (Invitrogen) according to the manufacture's protocols. About 500 ng of total RNA was added to 25 µl of Superscript III one-step RT-PCR mix (Invtrogen). Primers for PCR (forward, 5′- GTGTGCATTTAACGGGTGATAACAG: reverse, 5′-CGGAATTCATCCATCACGTGCTCCGGAAC) were specific two exons flanking the first intron to confirm unambiguously the PCR products from the *CrzR* cDNA template. To amplify 3' of *CrzR*, another primer set (forward, 5'-CATCTTCCATGTAGCGCGTGG: reverse, 5'- CATCGCCAGCTGCTCTCACACGCTC) was used, which were located on 3rd exon and 6th exon. For ALDH RT-PCR, the following primers (forward, 5'-ATGCTGCGCGTTTTGAAGACCGG: reverse, 5'-CTTCTTAGCCCGCTCCGCGCTG) were used. As a control for the amount of RNA, RT-PCR for *Drosophila β-tubulin* was performed in parallel (forward, 5′- GCAACAACTGGCCAAGGGTCATTAC: reverse, 5′-CTTGGCATCGAACATCTGCTGGGTCAG). To find optimal condition for comparison, we used three different cycles for amplification; 18 cycles, 20 cycles and 25 cycles.

Ethanol Intoxication test

Twenty male flies younger than 3 days old were collected from each cross, and kept in a food vial for 3-5 days. Well-fed flies were transferred to an empty 180 ml plastic fly stock bottle, which was laid a filter paper (ø 55 mm) at the bottom. The mouth was covered with 35 mm-plastic dish lid and sealed with parafilm. Ethanol solution (500 µl) of various concentrations was introduced to soak the filter paper using a syringe with a 20G 1½ needle. Completely intoxicated flies, which showed up-side down position, were counted every 2 min for 70 % ethanol or 5 min for 30% and 50% ethanol (Fig 4-3).

Recovery test from sedation

Twenty male flies younger than 3 days old were collected from each cross, and kept in a food vial for 3-5 days. The flies were transferred to an empty plastic vial, which was plugged with a cotton ball that had been soaked with 1 ml of 100% ethanol or 50μl of ethyl ether. After 15~20 min exposure to ethanol or 10 min to ethyl ether, flies were completely intoxicated or under anesthesia, then the soaked cotton ball was replaced with a fresh buzz plug. The number of fully recovered flies was counted every 10 minutes for up to 4 hours. Flies that were able to walk in a coordinated fashion and climbed the wall of the vial were considered 'fully recovered', while flies that did not wake up at 8 h after sedation deceased (Fig. 4-2).

Ethanol Content Assay

Twenty male flies were exposed to 1 ml of 100% ethanol in a plastic vial, as described previously. Flies were collected at 0, 10, and 20 min on dry ice to measure the amount of ethanol absorption. In a separate experiment, flies, which had been exposed to ethanol for 20 min, were transferred to ethanol-free environment, and then flies were collected after 30 minutes and 2 hours to check residual ethanol content. The frozen flies were homogenized in 300 μl of 50 mM Tris-HCl [pH 7.5] at 4℃ and spun down at the 13,000 g for 20 min at 4℃ (Heinstra et al., 1989; Lee et al., 2008). The NAD-ADH test kit (Sigma-Aldrich, St. Louis, MO) was used to determine the alcohol concentration according to the manufacturer's instructions with some modification for 96-well micro plate. Fly extract (4 μl) was mixed with 200 μl of enzyme solution in 96-

well micro plate and read the value at the 340 nm using Synergy™ 2 Multi-Detection Microplate Reader (BioTek Instruments, Winooski, VT). The ethanol contents were converted from percent to mM scale by manufacturer's protocol (Sigma-Aldrich).

Aldehyde dehydrogenase (ALDH) activity assay

Twenty male flies were homogenized in 300 µl of homogenizing buffer (50 mM-Na_2HPO_4/NaH_2PO_4, pH 7.4, 0.24 M sucrose, 0.5 mM EDTA, 0.5 mM dithiothreitol, 0.001% (w/v) phenylthiourea, and 1% (v/v) Triton X-100), as described previously (Heinstra et al., 1989). The homogenate was incubated for 15 min at room temperature and then centrifuged at 15,000g for 20 min at 4℃. Twenty five µg of total fly protein was mixed with 200 µl of aldehyde buffer (3.6 mM acetaldehyde, 1 mM β-NAD^+, and 2 mM pyrazole in 50 mM Tris buffer [pH 8.5]) in 96-well micro plate and read the value at the 340 nm using Synergy™ 2 Multi-Detection Microplate Reader. The pyrazole is inhibitor of the ADH activity, thus preventing any reaction product from ADH (Fry et al., 2004).

Generation of *Crz* mutant

To generate *Crz* mutant, we performed *P*-element mobilization (Robertson et al., 1988) from *P{EPgy2}pr-set7*^{EY04668}, that *P*-element was inserted in the 1st intron of *pr-set7* gene. The insertion position is ~500bp downstream of *Crz* transcription termination site. To supply the *P*-element transposase, male *P{EPgy2}pr-set7*^{EY04668} files were crossed with virgin *Δ2-3, Ki*. The male progeny, which had mosaic eye phenotype,

were crossed en mass with the third chromosome balancer virgins. *Ki*$^{+}$ male offsprings were collected and crossed singly with balancer again to generate mobilized stock (Fig. 4-8).

The homozygous wandering larvae were collected from each stock and purified genomic DNA using DNAzol. Briefly, 10-20 larvae were homogenized in 500 µl of DNAzol. The pellet was removed by centrifugation at 10,000g for 10 min at RT. The supernatant was transferred to a new tube and added 250 µl of absolute ethanol. DNA precipitation was performed by centrifugation at 5,000g for 5 min. The DNA was washed in 70% ethanol twice, and then dissolved in water. To confirm *Crz* mutation, *Crz* region was amplified by PCR using Crz 5´ end primer (5´-AGACGCAGTTGTGATTCGAAC) and Crz 3´ end primer (5´-AATTCATAAAAAATCCTGTGTT). To further confirm *Crz* mutation, Crz immunohistochemistry was performed.

IV. Results

Ethanol sensitivity of *Crz* cell deficient flies

In locust, CRZ injection induced phase-related change in the number of the antennal sensilla (Maeno and Tanaka, 2004). This phenotype suggests that *Crz* participates in the alcohol related response in *D. melanogaster* (Gibson and Oakeshott, 1981; Alcorta and Rubio, 1989). Thus we tested whether *Crz* is involved in alcohol perception.

Since *Crz* mutants were not available, we obtained flies devoid of *Crz* neurons (these flies are referred to as Crz-cell deficiency or Crz-CD for short). This was

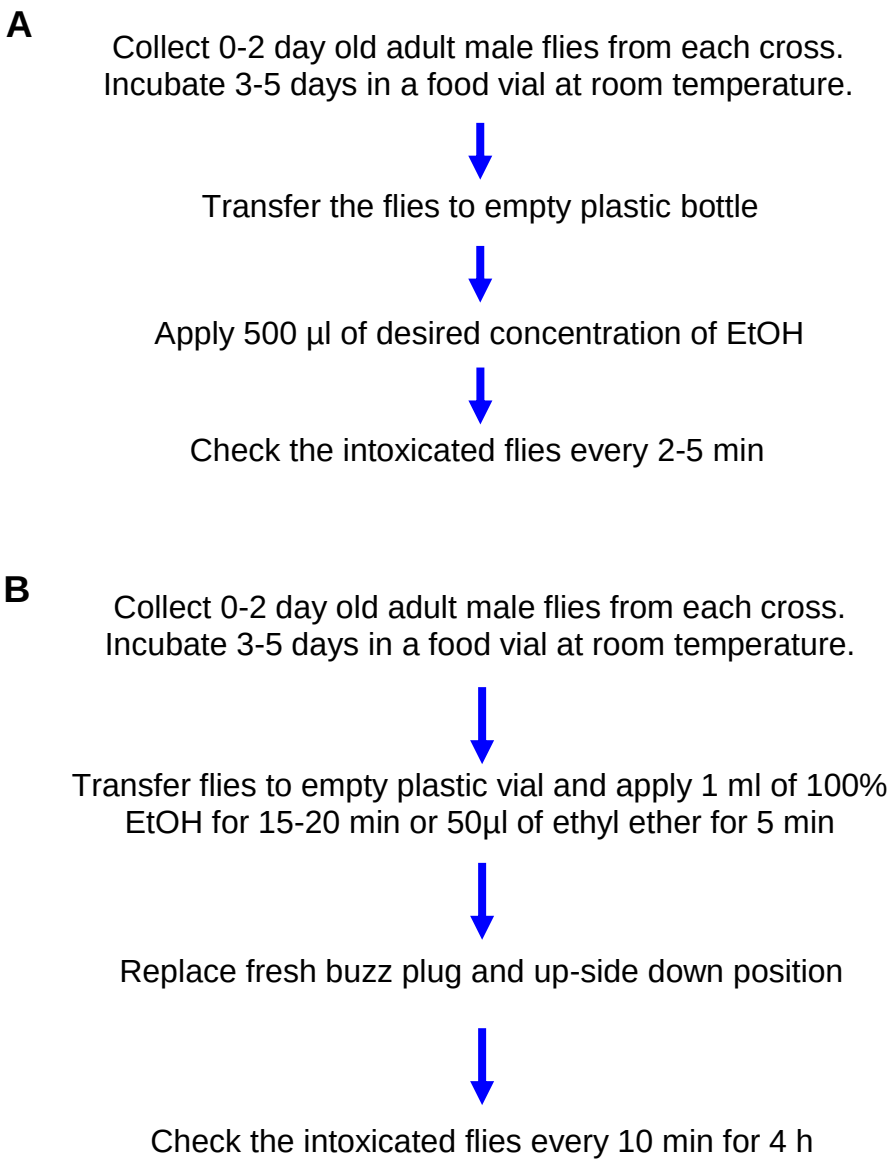


Fig. 4-2. Flow chart for fly alcohol intoxication (A) and recovery (B) test.

accomplished in the progeny derived from the crossing between *Crz-gal4* driver to a *UAS-hid*. During the course of this study, we found that Crz-CD flies showed significantly delayed recovery from ethanol-induced intoxication, which prompted us to examine this phenotype in a more comprehensive manner.

We first tested whether acute response to ethanol exposure was different between Crz-CD and controls. Synchronized flies (less than 10 days old, see also material and method) were exposed to desired alcohol concentrations. Flies were initially quite mobile and usually stay on the top of the vial: shortly after this state, they lost balance and control of their posture, and then fell to the bottom of the vial. These flies were considered intoxicated and counted at regular time intervals after they had been exposed to the ethanol vapor.

Rate of intoxication occurred in a dose-dependent manner. At 10 % ethanol solution, the flies showed very weak or no sedative effect even 2 h after exposure (data not shown). At 30 % ethanol, the flies were completely intoxicated in about an hour (Fig. 4-3 C). As expected, the response was much faster with higher ethanol concentrations, as the flies were completely sedated within 35-minutes exposure similarly to 70% and 50% ethanol (Fig. 4-3 A and B). The responses of the Crz-CD flies to ethanol vapor did not show any significant difference from those of control flies at all three ethanol concentrations tested. These results suggest that *Crz* does not affect acute response to ethanol-induced intoxication. Unexpectedly, npf-CD flies did not show more resistant in intoxication comparing Crz-CD flies (data not shown).

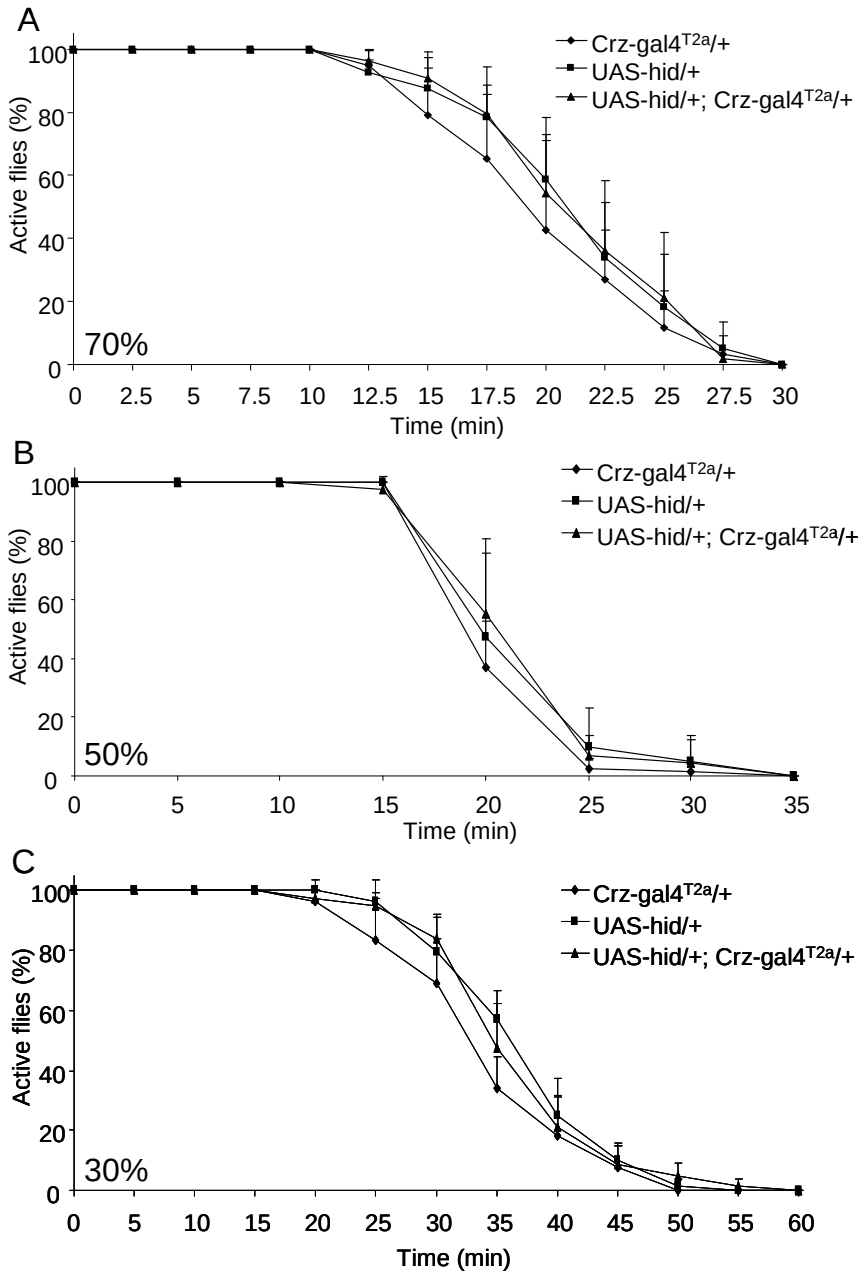


Fig. 4-3. Intoxication test for the desired concentration of ethyl alcohol. (A) 70% ethyl alcohol, (B) 50% ethyl alcohol, and (C) 30% ethyl alcohol. *Crz* expressing cell ablated group flies (triangle) and control group flies (diamond and rectangle) did not show any difference. The error bars indicate standard deviation. (n≥ 3)

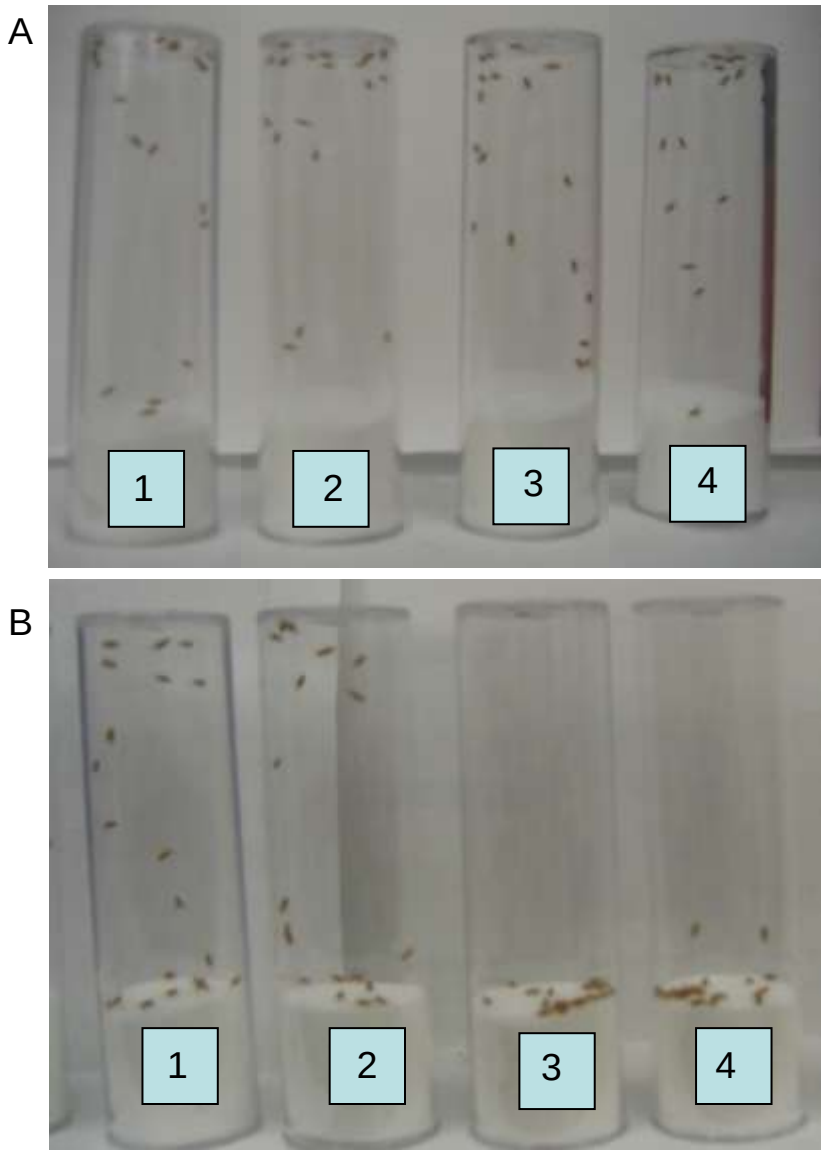


Fig. 4-4. Recovery test from ethyl ether- (A) and ethanol-induced sedation (B) at the 120 min after exposure. In ether sedation, almost all flies were recovered (A), but under ethanol sedation, very few Crz-CD and CrzR-KD flies were recovered. (1) *Crz-gal4*^{T2a}/+, (2) *UAS-hid*/+, (3) Crz-CD and (4) CrzR-KD

Recovery from ethanol sedation

Our preliminary observation indicated that *Crz* might be important for the recovery from ethanol intoxication (hangover). To further analyzed this type of phenotype, completely sedated flies from exposure to 100% ethanol for 20 minutes were transferred to ethanol-free environment, then flies that walked normally were counted.

Flies of control groups started to be recovered gradually starting from ~20 min in ethanol-free condition, and almost all flies returned to normal in 2 hours (Fig. 4-4). In contrast, flies lacking *Crz* neurons displayed significantly delayed recovery, as the recovery started at ca. 80 min. Furthermore, only 80% of flies were fully recovered after 3-4 hours and the remaining 20% were never awake, thus considered dead. Such hangover phenotype was also observed with different Crz-CD flies in which *Crz* cell death was induced by an ectopic expression of the *Dronc* caspase (Fig. 4-5).

We wondered whether the delayed recovery from ethanol-induced sedation of Crz-CD flies is specific to ethanol or a general response to other chemicals. To test this, Crz-CD fly was tested for the recovery from ethyl ether-induced sedation, which often used for fly anesthetization. We could not find any significant differences between Crz-CD fly and control flies (Fig. 4-6). Therefore, this result indicates that Crz-CD fly affects ethanol-related response.

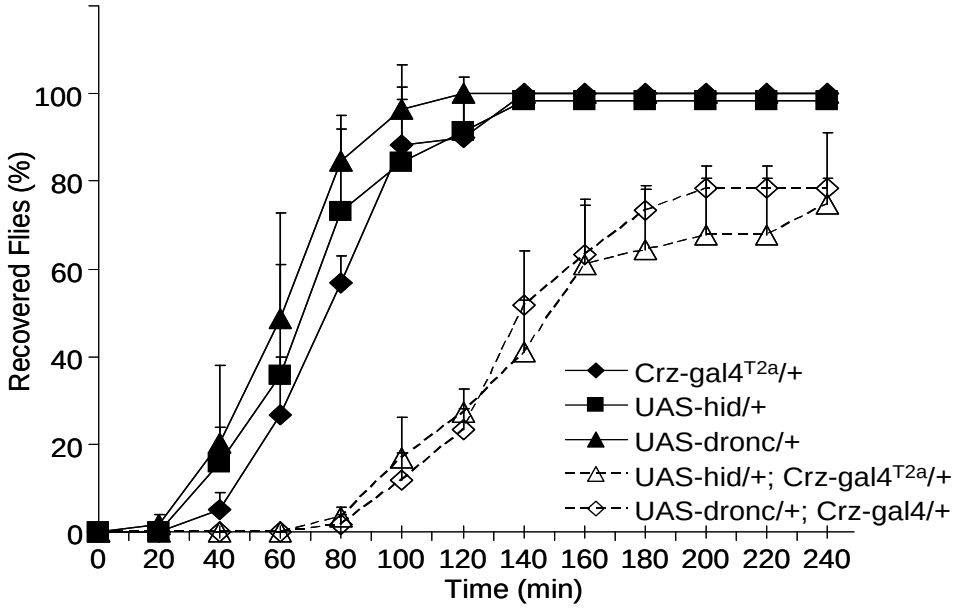


Fig. 4-5. Recovery test from ethanol-induced sedation. *Crz*-ablated flies (dashed lines, open symboles) showed delayed and incomplete recover compared to control group flies (solid lines, closed symbols) (n=3)

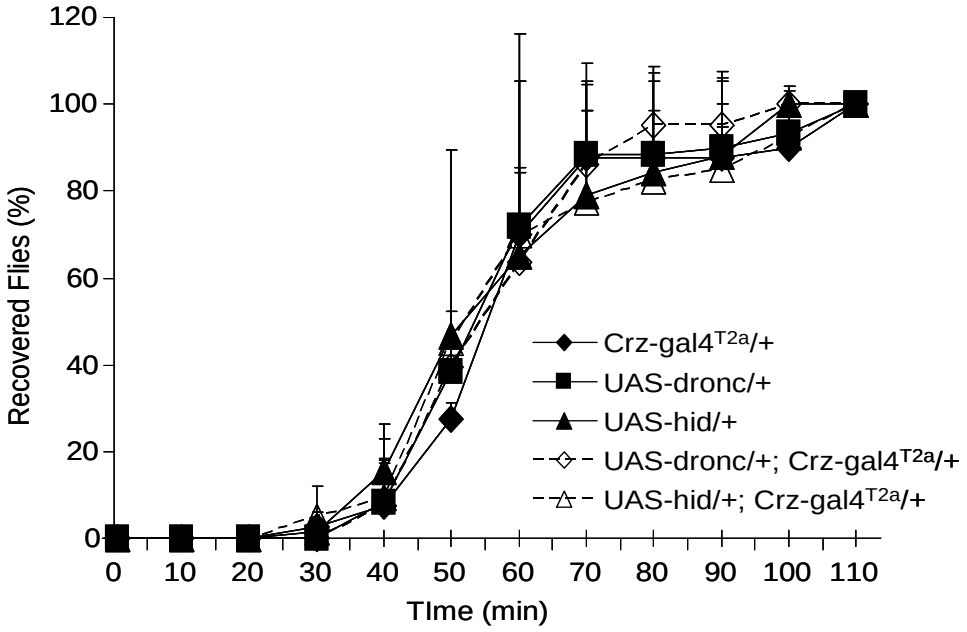


Fig. 4-6. Recovery test from ethyl ether-induced sedation. *Crz*-ablated flies (dashed lines, open symbols) and control group flies (solid lines, closed symbols) did not show any difference from sedation. (n=3)

Generation of *Crz* mutant fly

To further explore whether the hangover phenotype of Crz-CD is due to the absence of Crz production, we attempted to generate *Crz*-null mutant using RNAi-mediated gene silencing. To produce *Crz* mutant fly, we generated *Crz* inverted repeat (Crz^{ir}) construct and then introduced into host strain (*y w* mutant background, Fig. 4-7). In the result of transgenic lines, we expect to express transcripts with hairpin structure, which would mediate dsRNA-induced interference of *Crz* transcripts (Fig. 4-7). To verify *Crz* RNAi efficiency, we performed *Crz* immunohistochemistry in larval CNS. We tested total 6 different lines (at least 5 specimens per each line). As a result, the cell number and level of expression were comparable to *y w* control. Therefore, we concluded that double-stranded *Crz* RNA did not knockdown *Crz* expression *in vivo* (data not shown).

Alternative to RNAi, we performed *P*-element mobilization (Robertson et al., 1988) from $P\{EPgy2\}pr\text{-}set7^{EY04668}$. This transposon is inserted in the 1st introns of the *pr-set7* gene, which is ~500bp downstream of *Crz* transcription termination site (Karachentsev et al., 2005). We expected that *P*-element mobilization causes imprecise excision, deleting *Crz* coding region. Because *pr-set7* mutants are lethal at pupal stage, we collected, purified genomic DNA and performed PCR to amplify *Crz* coding region using homozygous wandering larva. From the first trial, we generated 110 independent mobilized lines, including 7 *y w* revertants. After PCR screening, we found two putative *Crz* mutants, which failed to produce PCR product. Further PCR analysis indicates that these lines carry a deletion extending to the *Crz* 3' UTR (Fig 4-9 A and B, Table 4-2). To verify *Crz* mutant, we performed immunohistochemistry. Unfortunately, those putative *Crz* mutant lines expressed proteins in all *Crz* neurons

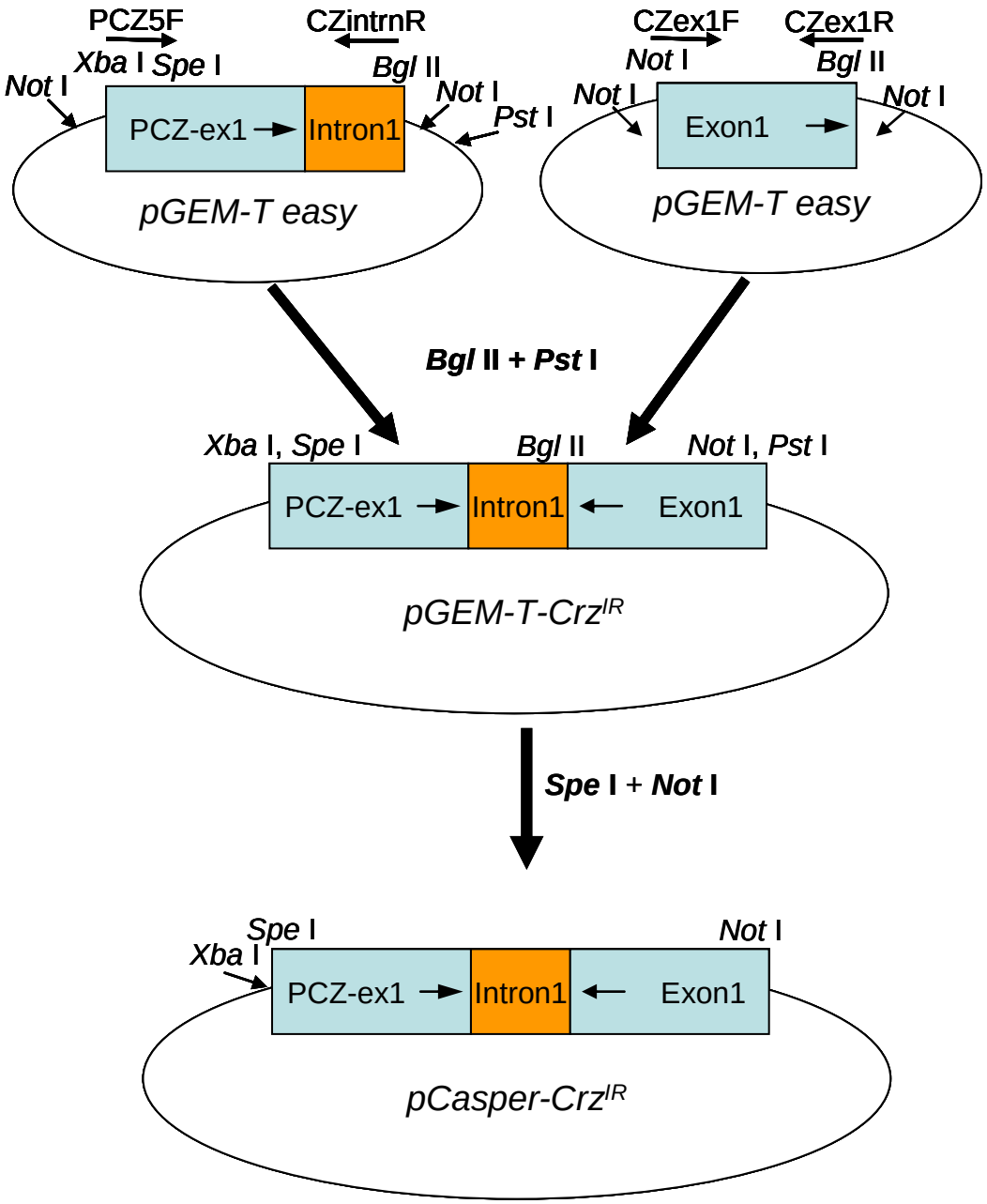


Fig. 4-7. Scheme of generating *Crz* RNAi construct.

(data not shown). From the second attempt, we established 80 individual lines including 28 *y w* revertants. PCR screening revealed two putative *Crz* mutants; both did not amplify *Crz* coding region using Crz 5' forward primer and Cz3 reverse primer set (data not shown). When PCR was performed using Crz 5' and Cz6 reverse primer, which is located on the 3' terminal of the first exon, both line produced PCR product. After Crz immunohistochemistry, we found that *Crz* immunoreactivity was intact; it indicates these putative *Crz* mutant lines produced mature Crz proteins, even though part of Crz-associated protein (CAP) region might be deleted (Fig. 4-9 C and D).

Generation of *Crz receptor* (*CrzR*) knock-down fly

Since we were unable to get *Crz*-null mutant, we turned our attention to the *Crz receptor* (*CrzR*). *D. melanogester CrzR* gene (CG10698) was isolated and characterized (CG10698, Alcorta, 1991; Cazzamali et al., 2002; Kim et al., 2004; Belmont et al., 2006). This G-protein coupled receptor (GPCR) has some degree of homology to *Drosophila adipokinetic hormones* (*AKH*) receptor and mammalian gonadotropin-releasing hormone receptor. To find expression pattern of *CrzR* in larval stage, we purified RNA from separate tissues and performed RT-PCR using *CrzR* specific primers. In larval stage, *Crz* receptor was highly expressed in the CNS and epidermis, and modestly in the fat body and tracheal. However, gut and Malphigian tubules expressed *CrzR* very weakly (Fig. 4-10).

Since no mutation is available for *CrzR*, we attempted to induce RNAi to generate *CrzR* knock-down flies. To do this, we subcloned from -59 to +328-bp fragment of *CrzR* cDNA in *sympUAS Drosophila* vector and introduced it into a w^{1118}

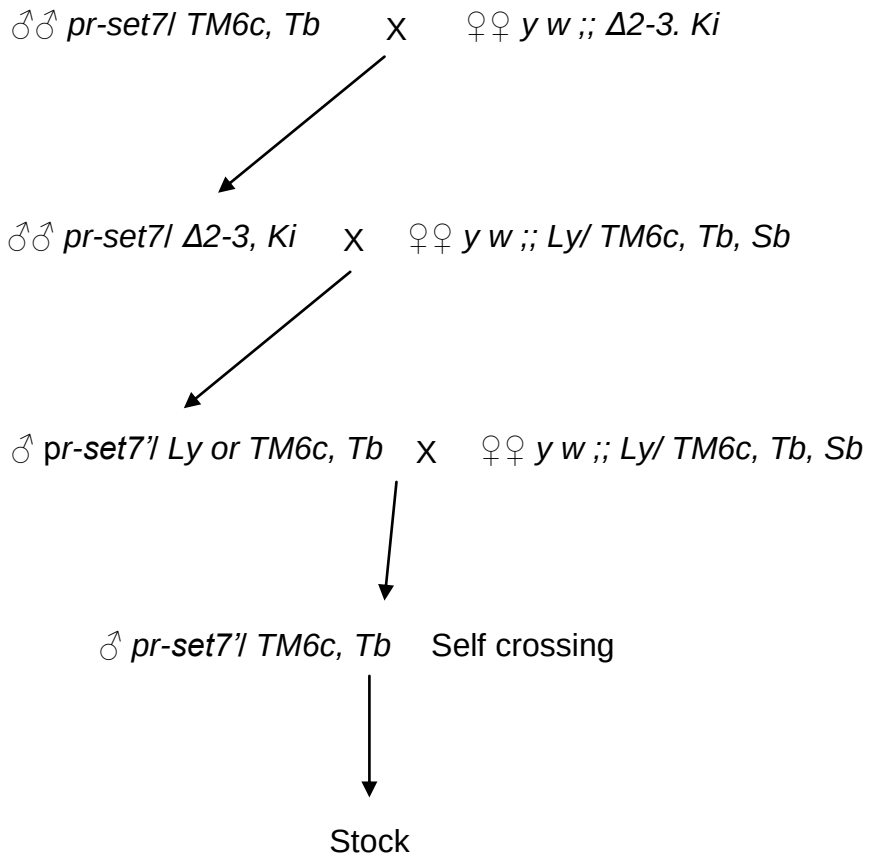


Fig. 4-8. Scheme of *P* element mobilization to generate *Crz* mutant.

Table 4-2. Summary of *P*-element mobilization to generate *Crz* mutant

Attempt	Total	y w revertant	Putative *Crz* mutant
1st	110	7	2
2nd	80	28	2

host. This transformation vector has yeast upstream activation sequences (*UAS*) flanking the target sequence to generate sense- and anti-sense RNA simultaneously, yielding double stranded RNA (Fig. 4-11, Giordano et al., 2002). To test efficacy of this construct, the transgenic flies (hereafter *UAS-CrzR*^{RNAi}) were crossed to universal *act-gal4* driver, and then RT-PCR was performed using RNA purified from the progeny. Twenty four individual transgenic lines were tested, from which two lines showed severe reduction of *CrzR* expression; these flies were used for further study (Fig 4-12).

Another putative *CrzR* mutant was generated by *Drosophila* gene disruption project. *GRHRII*^{MB00583} line, which is viable and fertile, was produced by *minos* transposable element inserted in the 5th intron of the *CrzR* gene (hereafter *CrzR*^{minos}, Bellen et al., 2004). RT-PCR using 5'-primers showed normal expression; however, 3'-primers did not produce PCR product, indicating that the mutant gene produce truncated transcripts (Fig. 4-11).

We tested whether *CrzR* knockdown and *CrzR*^{minos} mutation caused Crz-CD-like ethanol sensitivity. As shown for Crz-CD flies, CrzR knock-down (hereafter CrzR-KD) flies showed severely delayed recovery from ethanol sedation. After 4 h, less than 30 % of the CrzR-KD flies recovered (cf. 80 % recovery of Crz-CD flies Fig. 4-5; Fig. 4-13). This delayed and incomplete recovery is specific ethanol not ethyl ether sedation (Fig. 4-14). As previously mentioned, *CrzR* has strong homology to an AKH receptor; therefore *CrzR* might be activated by AKH. If *CrzR* is activated by AKH, more severe phenotype in CrzR-KD or *CrzR*^{minos} than Crz-CD might come from synergistic effect with AKH and Crz. To test this possibility, we measured recovery of AKH/ Crz double neuronal-cell ablation flies using ectopic expression of proapototic gene *hid* driven by

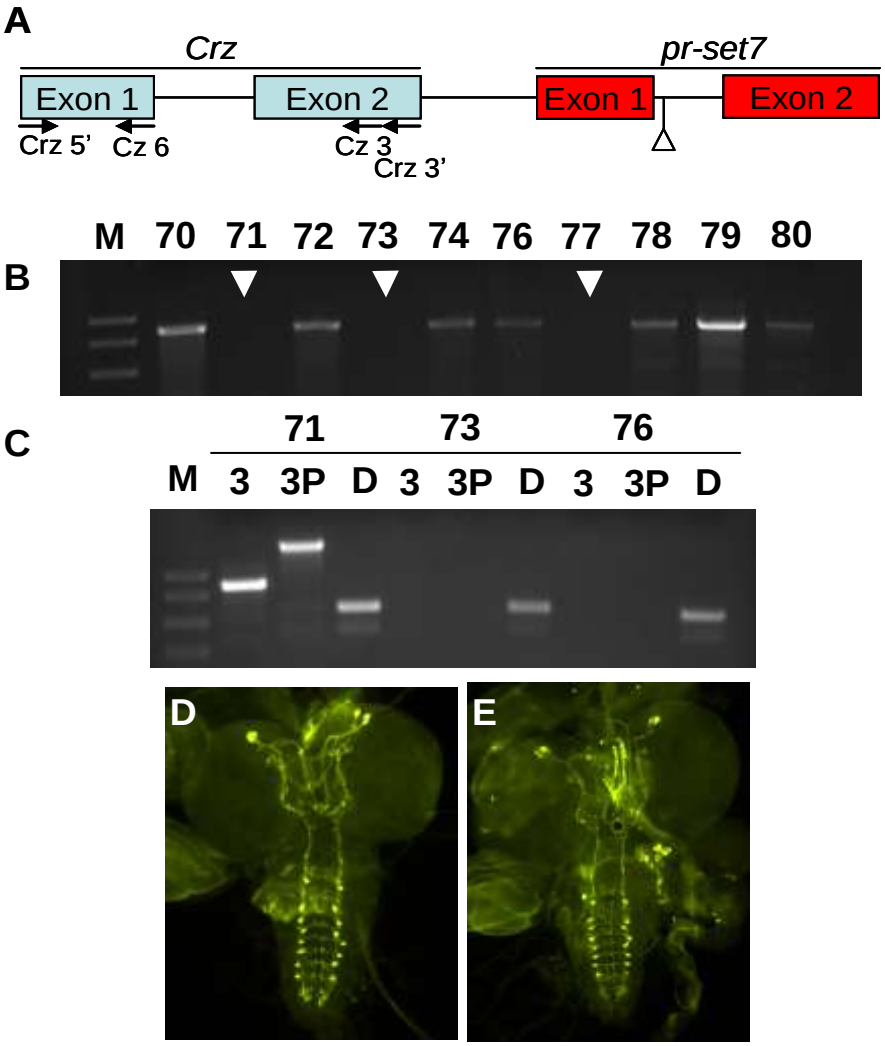


Fig. 4-9. Analysis of *P*-element mobilization. (A) Gene structure, location of primers and *P*-element insertion site. (B) PCR amplification to find putative *Crz* mutant. *Crz* 5' end and 3' end primer was used to amplify *Crz* genomic DNA. Three putative *Crz* mutants were found in (arrowhead). (C) Putative *Crz* mutants were reamplified using different primer sets (3: *Crz* 5' and Cz3, 3P: *Crz* 5' and *Crz* 3', D: *D. melanogaster* neuropeptide DSK primer as a control). The 73 and 76 lines failed to produce PCR product. (D, E). Immunohistochemistry in putative *Crz* mutant. The 73 (D) and 76 (E) lines produced mature Crz proteins.

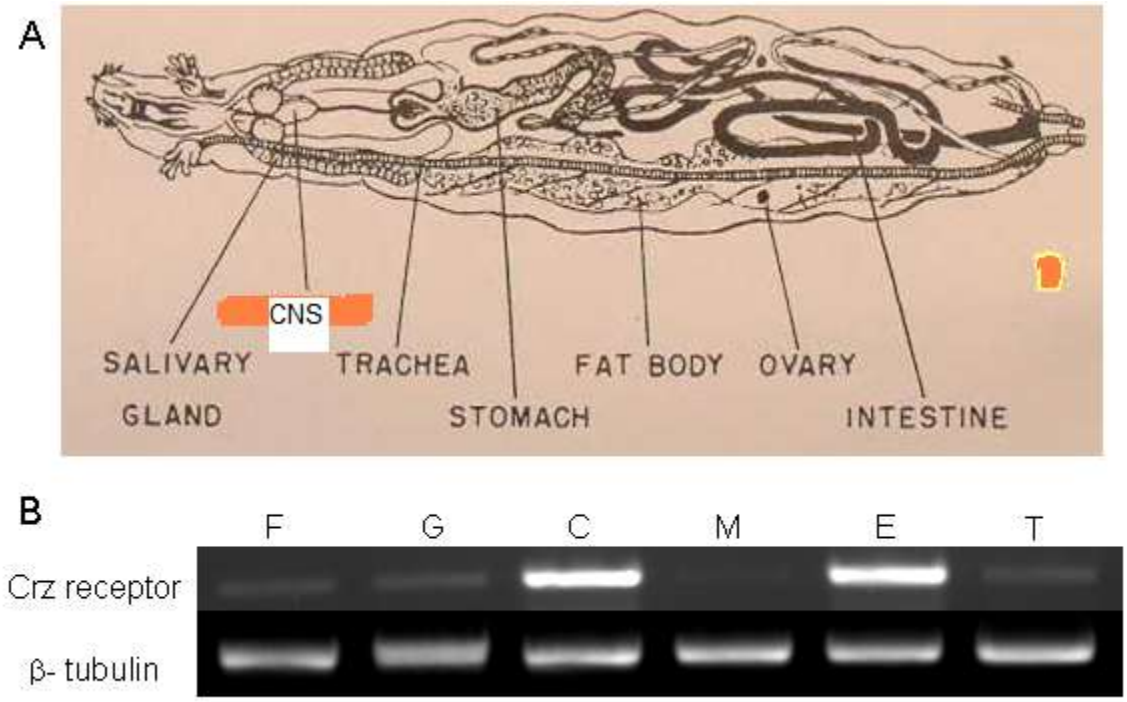


Fig. 4-10. *Crz receptor* (*CrzR*) expression in larval stage. (A) Dorsal view of larva (Adapted from Hartenstein, 1993). (B) *CrzR* was amplified by RT-PCR from different tissues of wondering larval stage. Central nervous system (line C) and epidermis and cuticles (line E) expressed highly *CrzR* mRNA. *β-tubulin* was used as a loading control. F, fat body; G, gut; M, Malphigian tubule; and T, trachea

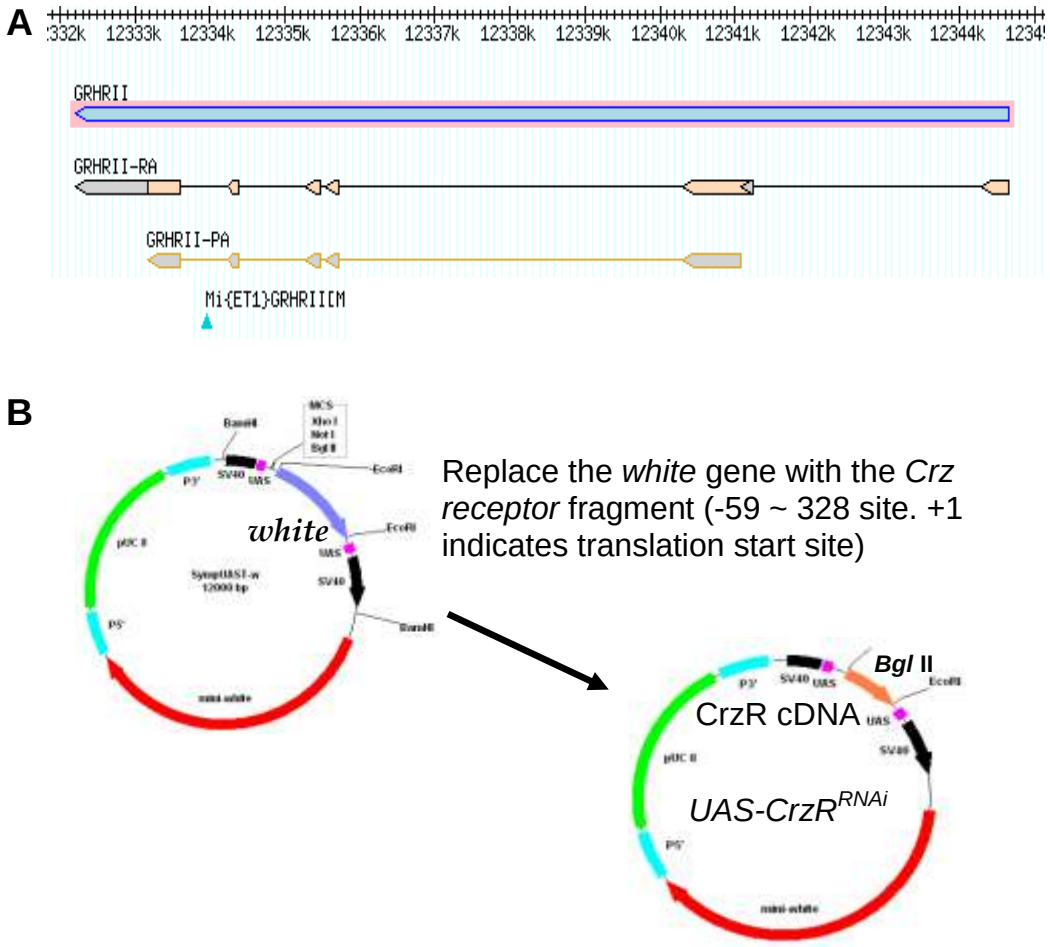


Fig. 4-11. *Crz receptor* (*CrzR*) gene structure and scheme of *UAS-CrzR*^{RNAi} construct. (A) Genomic DNA map of *CrzR* region. *CrzR* located on 69B on the left arm of the 3rd chromosome. The *minos* element inserted in 5th introns site (blue arrow). (B) Scheme of *UAS-CrzR*^{RNAi} construct. The mini *white* gene was replaced with partial *CrzR* cDNA in *SympUAST-w* transformation vector.

Akh/ *Crz-gal4* double driver. However, *Akh*/ *Crz* double neuronal cell ablation flies in recovery from ethanol intoxication is not significant different form Crz-CD flies. In contrast, *Adh* mutant flies (*Adh*^{fn23}) never recovered from sedation even after 4 h recovered time (Fig 4-13).

Ethanol metabolism and acetaldehyde dehydrogenase (ALDH) activity

It is possible that delayed recovery of Crz-CD and CrzR-KD flies might be caused by impaired metabolism of absorbed ethanol, thus rendering prolonged residual ethanol in the body. Ethanol is metabolized by two sequential enzyme activities: Alcohol dehydrogenase (ADH) and ALDH. To test this hypothesis, we undertook pharmacokinetic assay to measure ethanol content in the fly body. Interestingly, ethanol contents did not showed any significantly differences among genotypes (Table 4-3 and Fig. 15). After 20-min exposure to ethanol vapor when flies were completely intoxicated, ethanol body concentration reached around 22 mM; but within 2 hours after recovery, ethanol concentration was reduced to basal lines. However, Crz-CD flies were still hung-over and sedated (Fig. 4-5). These data suggest that Crz-CD flies have ethanol absorption rate similar to control groups.

Acetaldehyde is 30 times more toxic than ethanol; therefore, converting acetaldehyde to less-toxic acetate is more important step in ethanol metabolism (Sprince et al., 1974). In mammals, this step of ethanol metabolism is mediated by aldehyde dehydrogenase (ALDH, Weiner, 1982, 1987). Fly has one ALDH encoding gene (Heinstra et al., 1989), and we measured ALDH activity using enzyme assay. About 25 µg of total protein was mixed with ALDH enzyme assay solution, which

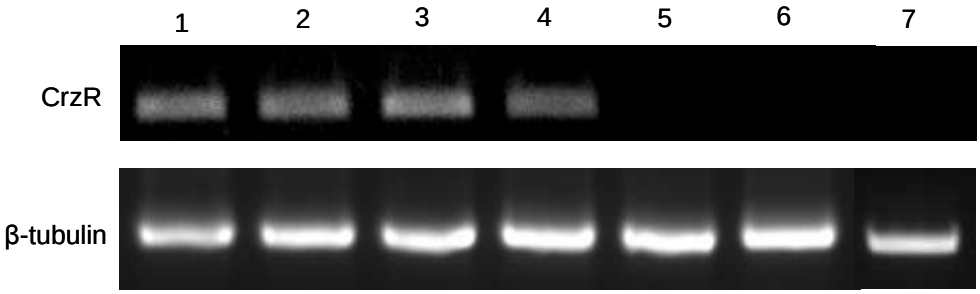


Fig. 4-12. RT-PCR results show that *CrzR* mRNA levels were significantly reduced.

(1, *y w*; 2, *act-gal4*/+; 3, *UAS-CrzR*^{RNAiS3S}/+; 4, *UAS-CrzR*^{RNAiT17}/+;

5, *UAS-CrzR*^{RNAiS3S}/+; *act-gal4*/+; 6, *UAS-CrzR*^{RNAiT17}/ *act-gal4*; and 7, *CrzR*^{minos})

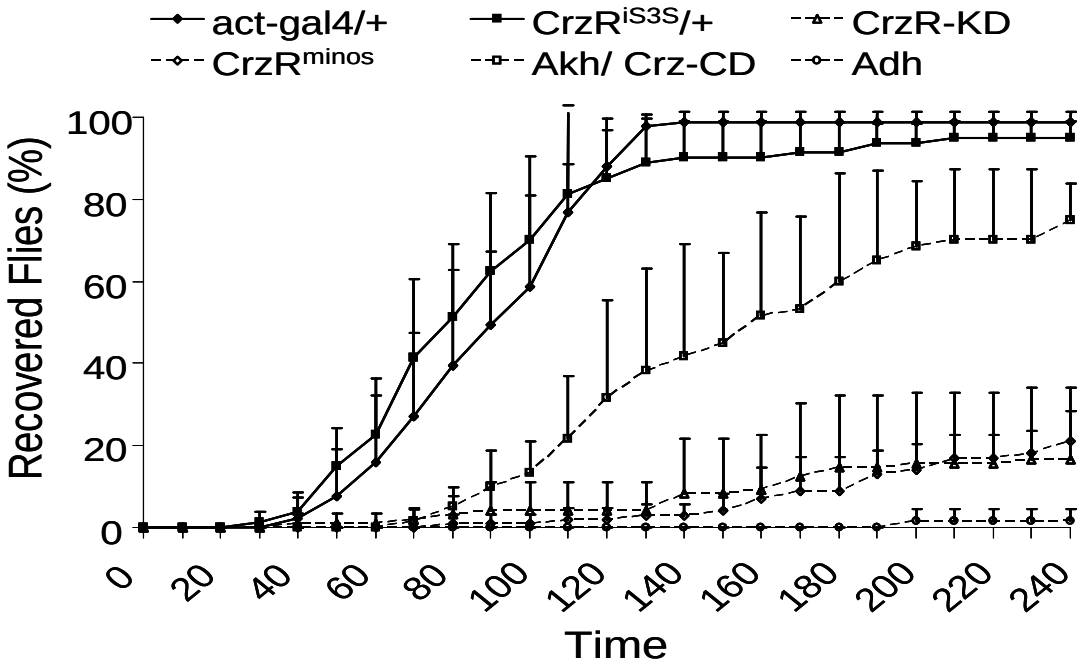


Fig. 4-13**.** Recovery from ethanol-induced sedation in CrzR-KD flies. CrzR-KD and *CrzR*^{minos} flies (dashed lines, open triangle and diamond) showed significantly delayed recovery from ethanol sedation, while control groups (solid lines, closed symbols) fully recovered 2 h after ethanol exposure. CrzR-KD and *Crz*^{minos} mutant showed intermediate recovery between *Crz*, *adipokinetic hormone* (*Akh*) double cell deficiency and *Adh*^{fn23}. *Adh*^{fn23} flies never recovered from sedation. *Crz*/ *AKH*-double cell deficiency does not show any difference comparing to Crz-CD flies. (n≥ 3)

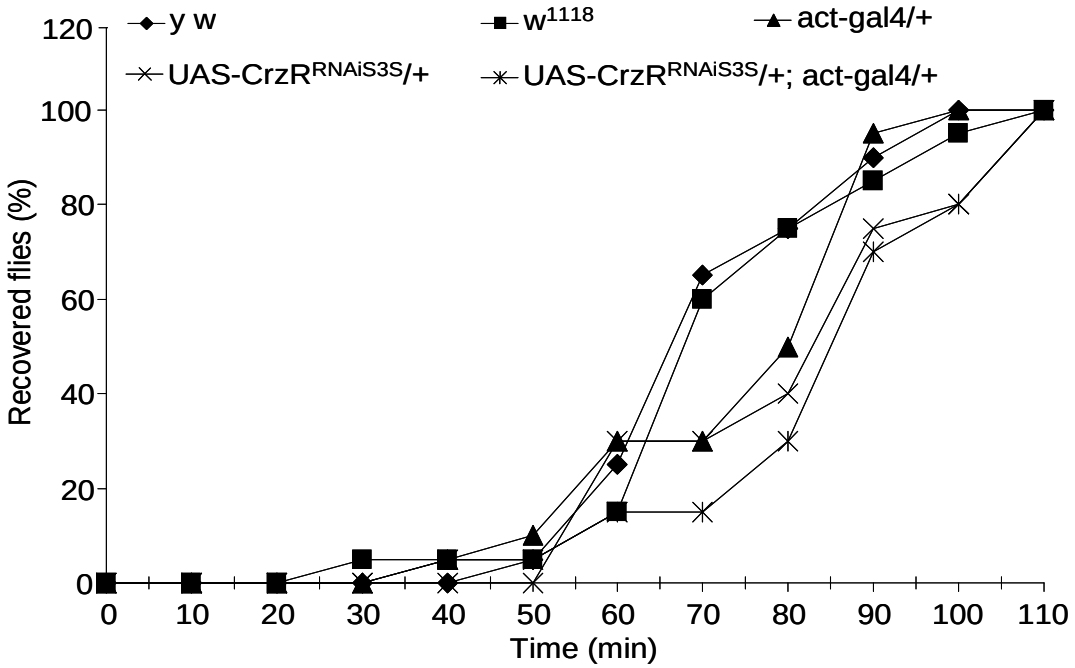


Fig. 4-14. Recovery from ethyl ether-induced sedation in CrzR-KD flies. CrzR-KD flies (open symbols) and control group (closed symbols) did not show any difference from sedation (n=1).

included 3.6 mM acetaldehyde as a substrate, 1 mM NAD+ and 0.2 mM pyrazole to block the ADH activity. Every 5 minute, absorbance was measured at the 340 nm. Intriguingly, ALDH enzyme activity in Crz-CD and *CrzR*^{minos} flies was reduced to ~60 % of control groups (Fig 4-16 B and Table 4-4). ALDH transcript level was detected by RT-PCR. Transcript level was quite similar among the control group, Crz-CD, CrzR-KD and *CrzR* mutant (*CrzR*^{minos}, Fig. 4-16 A). This result strongly suggested that delayed recovery from ethanol sedation in Crz-CD and CrzR-KD flies was caused by low ALDH activity, which might come from accumulation of acetaldehyde.

Discussion

In this report, we have first identified that the neuropeptide *Crz* signal transduction pathway involves in a modulation of ethanol metabolism in *D. melanogester*. Ethanol related response and metabolism is heavily studied because ethanol is used and consumed as beverage and drug, but our knowledge of the mechanisms by which it controls brain activity and behavior is still unknown. Although proteins in ethanol metabolism are quite different during evolutionary and structurally, metabolic pathway is highly conserved between mammals and fruit flies (Hinson and Neal, 1972; Bellen, 1998; Moore et al., 1998). In contrast to mammals, *Drosophila* has only one ADH and mitochondrial ALDH gene. Therefore, we are trying to use *D. melanogester* as a model system for understanding of mechanism of alcohol metabolism (Wolf and Heberlein, 2003). Many fruit flies, including *D. melanogester*, well develop alcohol metabolism, because they live on fermenting fruit, where ethanol concentration reaches several percent (McKenzie and McKechnie, 1978; Gibson and

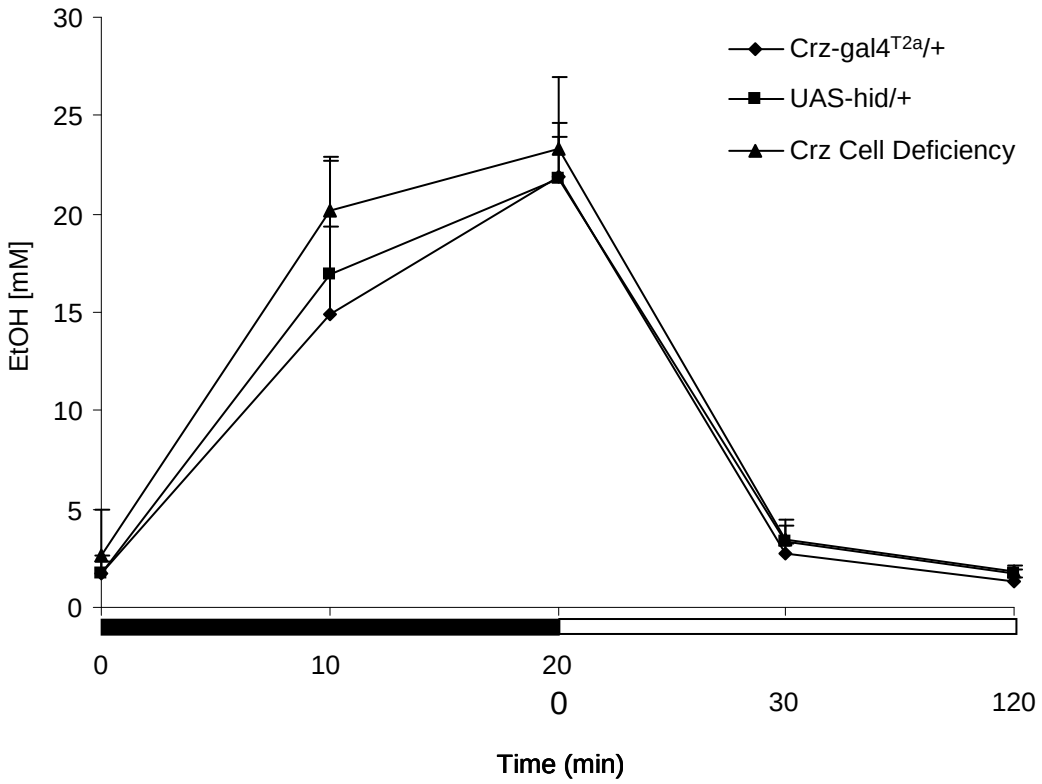


Fig. 4-15. Crz-CD flies showed normal ethanol absorption and degradation. To measure ethanol absorption, the concentration of body ethanol content was determined in flies when they exposed to 1 ml ethanol for 0, 10, and 20 min (black box). Remaining ethanol body content was measured for 30 and 120 min after 20 min ethanol exposure. Crz-CD flies showed similar concentration of ethanol at the specific time point. (n=5)

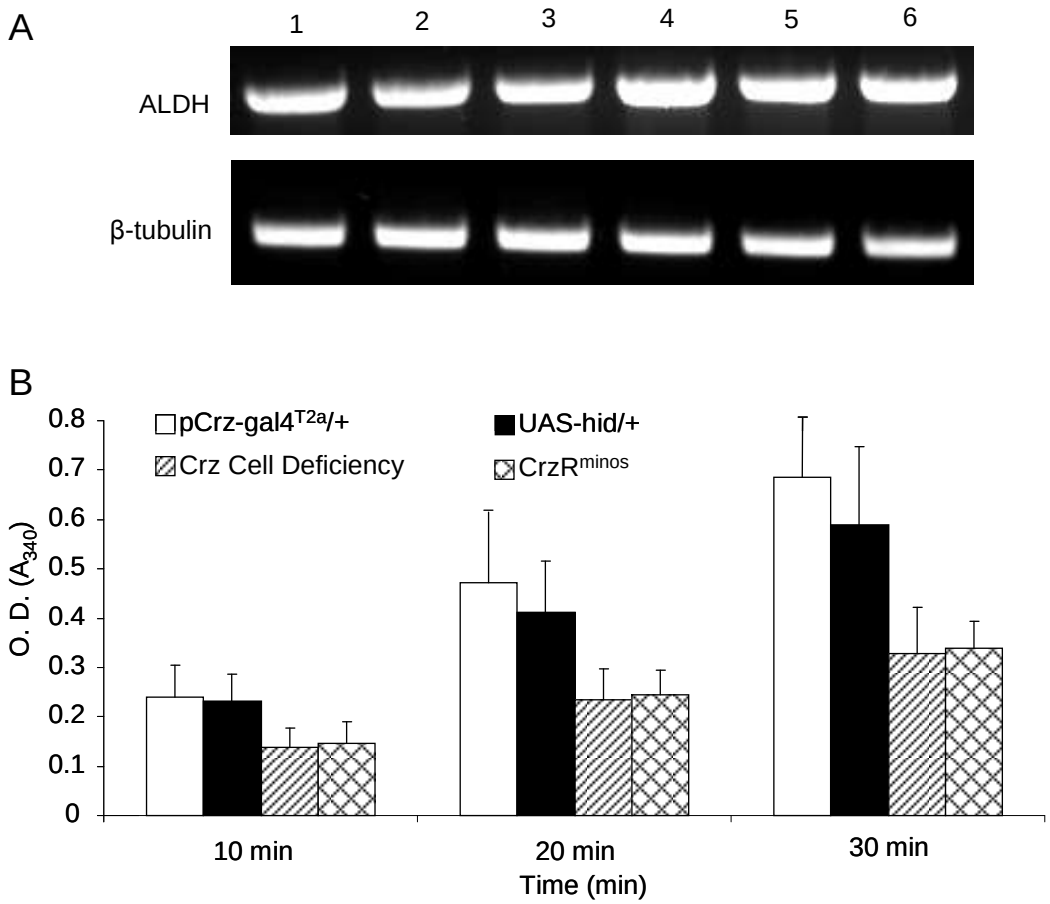


Fig. 4-16. Crz controls ALDH enzyme activity. (A) RT-PCR results showed *Crz* does not affect *Aldh* transcription levels. *β-tubulin* used as a loading control.

1, *Crz-gal4*^{T2a}/+; 2, *UAS-hid/*+; 3, Crz-CD; 4. *UAS-CrzR*^{RNAiS3S}/+;

5, *UAS-CrzR*^{RNAiS3S}*/+; act-gal4*/+; and 6, *CrzR*^{minos} (B) ALDH enzyme activity was reduced in Crz-CD and *CrzR*^{minos} flies (n=8).

Oakeshott, 1981). Previously, Heinstra group insists that more than 75% of acetaldehyde is converted to acetate by ADH in larval stage (Heinstra et al., 1989). Contradictory, Fry et al found that *Drosophila* ALDH is more important in ethanol metabolism; when they incubated the flies in ethanol supplemented media, ALDH activity was increased comparing incubated in normal media group (Fry et al., 2004). In addition, *Aldh* mutants was intoxicated in a concentration of ethanol, while is tolerate in this concentration wild-type (Fry and Saweikis, 2006).

Crz-CD, as well as Crz-KD and *CrzR*^{minos} flies showed significantly delayed recovery phenotype from ethanol sedation (Fig 4-5 and Fig. 4-13). In contrast to a memory mutant *amnesiac* (*amn*), Crz-CD fly did not affect ethanol sensitivity (cf. Moore et al., 1998). Interestingly, ethanol absorption and metabolism did not change in Crz-CD fly (Fig. 4-15 and Table 4-3); however, ALDH activity was significantly reduced in Crz-CD, CrzR-KD and *CrzR*^{minos} (Fig. 4-17). In other word, low activity of ALDH causes accumulation of acetaldehyde in the body, and leads fly hangover or lethal effect. We suggest two possibilities. One is aldehyde transport problem form cytoplasm to mitochondria. As previously mentioned, *Drosophila* has one ALDH protein in mitochondria. For the conversion from aldehyde to acetate, cytosolic aldehyde needs to transfer to the mitochondria. However, the transport system did not work properly, ALDH may not access to its substrate and build increased aldehyde concentration. Another possibility is *Crz* involving the control of ALDH enzyme activity. ALDH needs NAD^+ as a cofactor for its activity. If *Crz* regulates cofactor-binding on ALDH active pocket, Crz-CD or CrzR-KD flies shows weak enzyme activity. Although we do not still

understand the mechanism, *Crz* does not control *Aldh* expression, but controls ALDH enzyme.

Table 4-3. Fly body alcohol contents [mM]

genotype	Absorption [n=5]			Degradation [n=5]	
	0 min	10 min	20 min	30 min	120 min
Crz-gal4^{T2a}	1.77±0.82	14.86±4.54	21.88±2.05	2.69±0.51	1.28±0.21
UAS-hid	1.76±0.54	16.89±5.84	21.77±2.89	3.33±0.81	1.72±0.17
Crz-CD	2.62±2.31	20.15±2.73	23.34±3.60	3.43±1.05	1.86±0.26

Table. 4-4. ALDH activity test (A_{340}, per 25 µg total protein)

Time	Crz-gal4^{T2a} (n=8)		UAS-hid (n=8)		Crz-CD(n=8)		CrzR^{minos} (n=7)	
(Min)	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
10 min	0.241	0.065	0.231	0.055	0.138	0.039	0.145	0.045
20 min	0.472	0.142	0.411	0.105	0.235	0.063	0.244	0.050
30 min	0.686	0.231	0.590	0.159	0.329	0.094	0.340	0.054

Chapter Five

Can I Smell It? Function of *Corazonin* in Olfactory Response

I. Abstract

Odor perception is the basic instinct for survival. In locust, CRZ injection affected antennal sensilla development, suggesting that *Crz* affects animal's chemical perception. To explore the roles of *Crz* associated with chemosensory in *Drosophila*, we tested odor perception of Crz-CD and CrzR-KD flies. These flies showed significant defect in finding the source of complex volatile molecules derived from yeast paste compared to control flies. With a single molecule, Crz-CD and CrzR-KD flies showed delayed responses. However, Crz-CD and *CrzR* mutation did not affect trichoid antennal sensilla development.

II. Introduction

The olfactory cues provide insects with information about the locations of food, mates, and oviposition sites. The olfactory system of insect and mammals exhibit many similarities, suggesting that the cellular and molecular mechanisms underlying chemosensory system may be shared. Because of this, the insect olfaction system has garnered increasing interest. In *Drosophila*, attractive and repulsive compounds have been studied (Alcorta and Rubio, 1989).

The initial steps in odor detection involve the binding of a volatile odor to odorant receptor (OR) proteins displayed on ciliated dendrites of specialized olfactory receptor neurons (ORNs; Hallem and Carlson, 2004a, 2004b; Hallem et al., 2004). The OR genes are expressed in subpopulations of ORNs on the 3rd antennal segment and maxillary palps in *Drosophila* (Clyne et al., 1999a, 1999b; Larsson et al., 2004). The OR genes are members of a rapidly diverging superfamily of insect chemosensory genes that encode new family of seven transmembrane domain receptors with no homology to nematode or vertebrate ORs (Hill et al., 2002). The odor response profile of a given ORN is governed by the selective expression of one or more members of the family of 61 OR. One member of the OR gene family, *Or83b*, is strikingly different from other OR genes. Unlike the conventional ORs, it has clear homologs in other insect species that share nearly 70% amino acid identity (Hill et al., 2002; Pitts et al., 2004). *Or83b* and its homologs in other insects are coexpressed and formed heterodimer with conventional ORs in a large proportion of ORNs (Pitts et al., 2004; Pitts and Zwiebel, 2006).

Recently, a new family of odorant receptors was identified. These receptors share ligand binding domain of Ionotropic glutamate receptors (iGluRs), which are important for glutamate binding and opening ion channel (Mayer and Armstrong, 2004). In microarray screening, six iGluR-related genes, which named ionotropic receptors (IRs), are expressed in the antenna in *Drosophila*. These ionotropic receptors are expressed in coleoconic sensilla with *OR35a* and *OR83b*; however, IRs are not expressed in basiconic and trichoid sensilla. Misexpression of IRs in different ORNs is

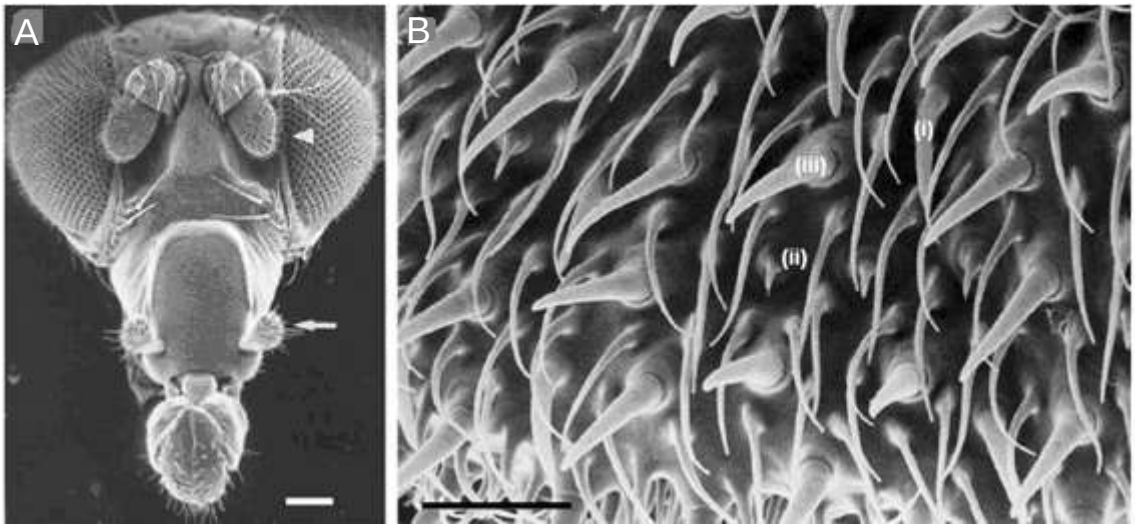


Fig. 5-1. The *Drosophila* olfactory organs. (A) The adult head with antennae (arrow head) and maxillary palp (arrow). Scale bar indicates 100 µm. (B) Scanning electron microscopy (SEM) for morphological types of *Drosophila* olfactory sensilla. (i) basiconic, (ii) coleoconic and (iii) trichoid. Scale bar indicates 10 µm. Figure adopted from Hallem and Carlson, 2004.

sufficient to induce ectopic olfactory sensitivity (Benton et al., 2009; Spletter and Luo, 2009).

In *Drosophila*, about 1300 ORNs exist in the antenna and maxillary palps on both sides of the head and project axons to the antennal lobe where they terminate in ~43 morphologically individual and entangled synapse modules known as glomeruli. ORNs that express the same OR, although distributed across the surface of the antenna and maxillary palps, project their axons to the same glomerular target in the antennal lobe. There, they are thought to form excitatory synapses with at least two classes of neurons, the local interneurons (LNs) and the projection neurons (PNs). The axonless LNs are thought primarily to be GABAergic inhibitory neurons, and have broad, multiglomerular ramifications within the antennal lobe. A unique feature of the circuitry within the insect antennal lobe is the existence of reciprocal dendrodendritic connections between the PNs and the LNs. The presence of these unique junctions with both transmissive and receptive synapses provides anatomical evidence that each glomerulus makes computations that may underlie odor perception, discrimination, and learning, rather than being a simple transit station for the throughput of olfactory information. Individual PNs generally extend dendrites into a single glomerulus and then convey the processed olfactory information to the 3˚ olfactory neurons (Fig. 5-2; Davis, 2004; Keene and Waddell, 2007). Such hierarchical odor processing system is remarkably conserved, thus validity between mammals and insects, the study of *Drosophila* olfactory system as a model human odor recognition and response (Fig. 5-3; Davis, 2004).

We previously have shown *Crz* neurons in the pars lateralis which is in close vicinity of the Lateral horn. Thus we investigated whether *Crz* has a function associated

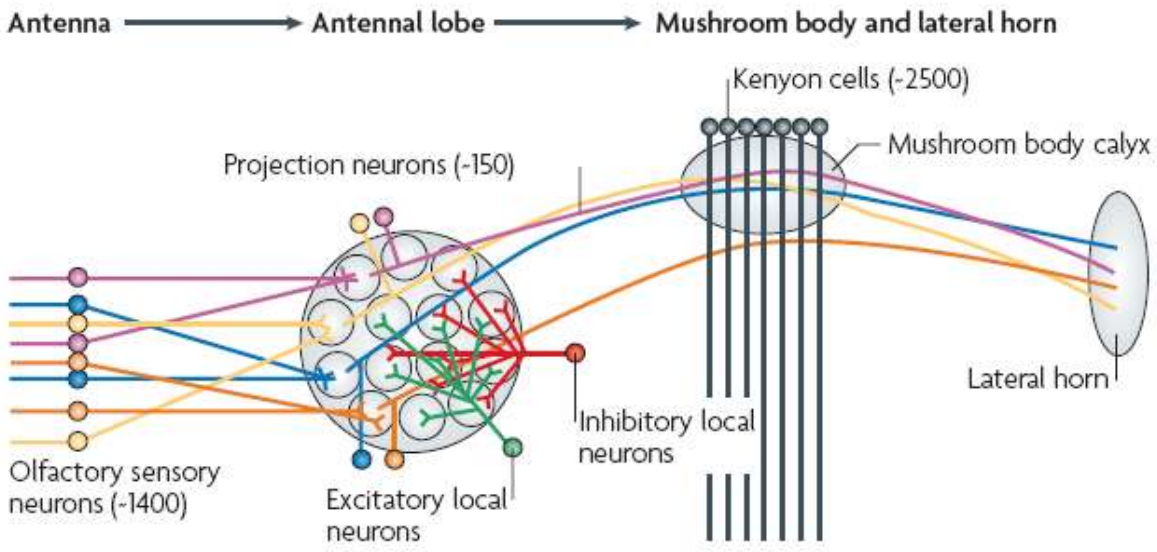


Fig. 5-2. Schematic cartoon of the *Drosophila* adult olfactory circuit. ORNs expressing the same olfactory receptor (same color) project their axons to the same glomerulus in the antennal lobe. On the antennal lobe, three types of interneuron are process the olfactory signals and transfer it to Kenyon cells in mushroom body calyx and lateral horn. Figure adopted from Keene and Waddell, 2007.

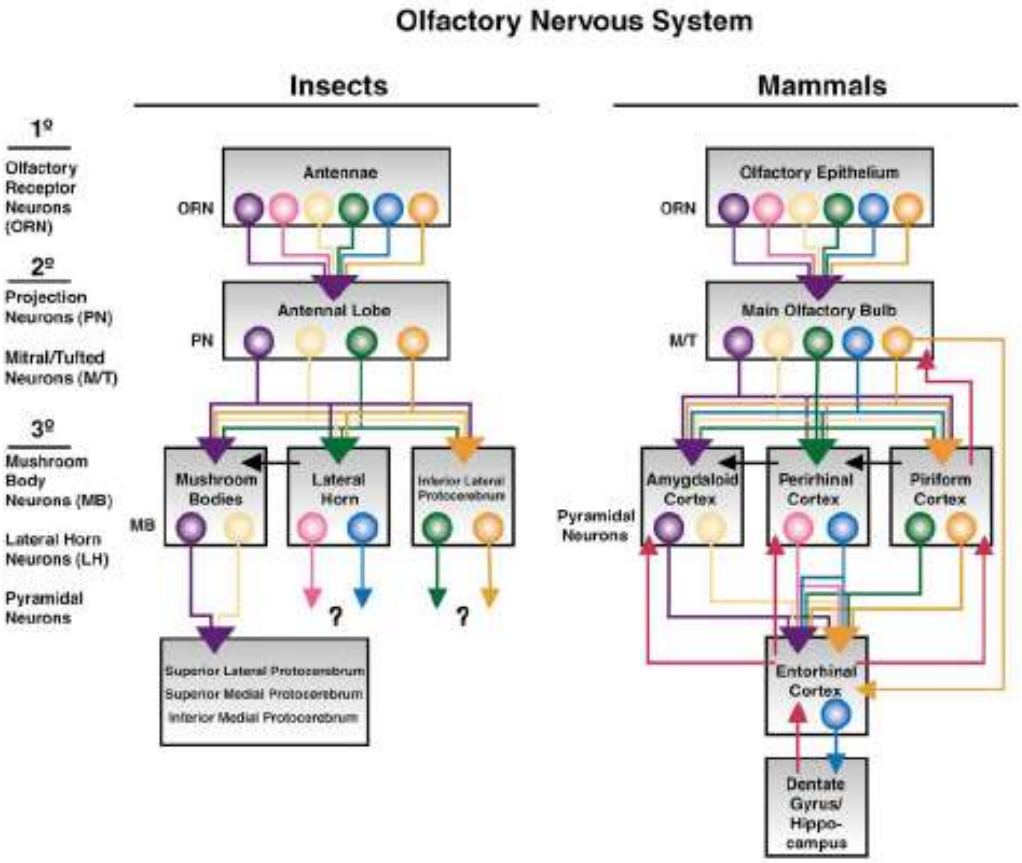


Fig. 5-3. Conserved mechanism of odor perception in insects and mammals. Figure modified from Davis, 2004.

with chemical sensory system. Here, we showed *Crz* neurons participate in chemical perception in *Drosophila*.

III. Materials and Method

Fly Olfactory Trap Assay

Fifty male flies younger than 3 days old were collected from each cross, and kept in a corn meal food vial for 3-5 days in constant darkness. Well fed flies were transferred to an empty vial which had been covered with 1% agarose to supply flies with water. After 12-hour starvation, the flies were transferred to an empty vial and immobilized in ice. The immobilized flies were transferred to a Petri dish (100 X 15 mm) which had been overlaid with 1% agarose and put trap which made of micro centrifuge tube and two yellow plastic micropipptte tips (Alcorta and Rubio, 1989; Woodard et al., 1989; Larsson et al., 2004a). The experiment was performed in darkness at room temperature to minimize any visual input. We checked numbers of flies entrapped every 12 h for up to 48 h under dim red light. The attraction index was calculated as follows: (Number of flies in attractant trap– Number of flies in water trap) / (Total number of flies at the starting point – Number of dead flies) X 100 (%)

For odorant preparation to test single chemical, various concentrations of odorant were mixed with melted 0.5% agarose at 50℃. The agarose was applied to the lid of 1.5 ml micro centrifuge tube up and waited to harden. To prepare yeast paste, 10g of dried yeast granule was mixed with 10 ml milli Q water, and then filled ~300 µl of the yeast paste on the lid of effendorf tube.

Count of trichoid sensilla on the antennae

The male antennae were dissected in isopropyl alcohol, and mounted on the slide with Permount. Individual antenna was taken pictures at several different focal planes. The trichoid antennal sensilla were counted.

Longevity Test

Flies were raised on a standard cornmeal-yeast-agar medium. Adult flies were collected under CO_2 anesthesia (20 male flies per vial) and onto 10% sugar/yeast food (10% sucrose, 10% yeast granule; 1% agar and 0.025% Methy-4-hydroxybenzoate). Food vials were changed every three days and dead flies were removed and counted. Flies were maintained in a 12:12 light-dark (LD) cycle, as well as constant temperature (29ºC) and relative humidity (60%).

IV. Results

Crz-CD flies are insensitive to yeast paste

To explore a relationship between *Crz* and chemoperception, we performed olfactory trap assay (Fig.5-4 and 5-5). Yeast is an important food source for *Drosophilidae* and chemicals producing from yeast are strong attractant for *D. melanogaster* (Shehata et al., 1955; Pavan et al., 1957; Hollingsworth and Burcombe, 1970; David and Van Herrewege, 1971). Thus we used yeast paste as an attractant. As previously described in chapter IV, we used Crz-CD flies for olfactory trap assay. Flies were given 12 hours fasting period to possibly enhance their motivation for finding

odor food sources. When we tested without starvation, flies response was delayed

Collect 0-2 day old adult male flies.

Incubate 3-5 days in constant darkness in a general food vial.

↓

Transfer the flies to a vial containing 1% agarose gel and starve for 12 hours.

↓

Apply starved flies to 100mm Petri dish covered with 1% agarose gel.

↓

Check the entrapped flies every 12 hours for up to 48 hours.

Fig. 5-4. Flow chart for fly olfactory trap assay.

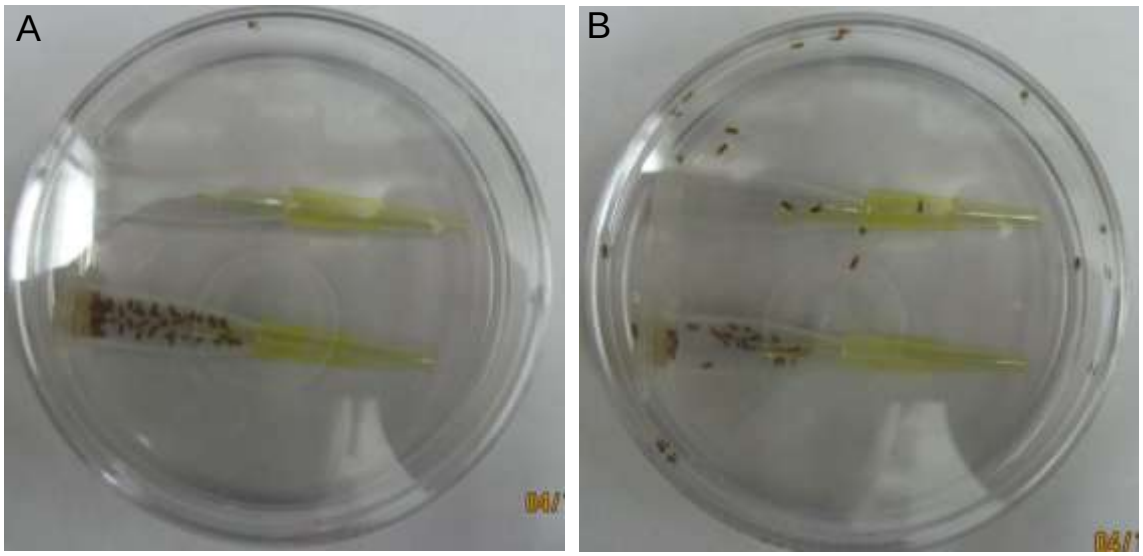


Fig 5-5. Olfactory trap assay. Fly olfactory entrap assay in *Crz receptor* (*CrzR*) knock down (CrzR-KD) flies by *CrzR*-RNAi construct using yeast paste as an attractant. After 48 hours, more than 80% of control flies (*y w*; *UAS-CrzR*^{RNAiS3s}/ +) were entrapped into the yeast paste trap (A). However, between 40% and 50% of CrzR-KD flies (y w; *UAS-CrzR*^{RNAiS3s}/+; *act-gal4*/+) were entrapped into the yeast paste trap (B)

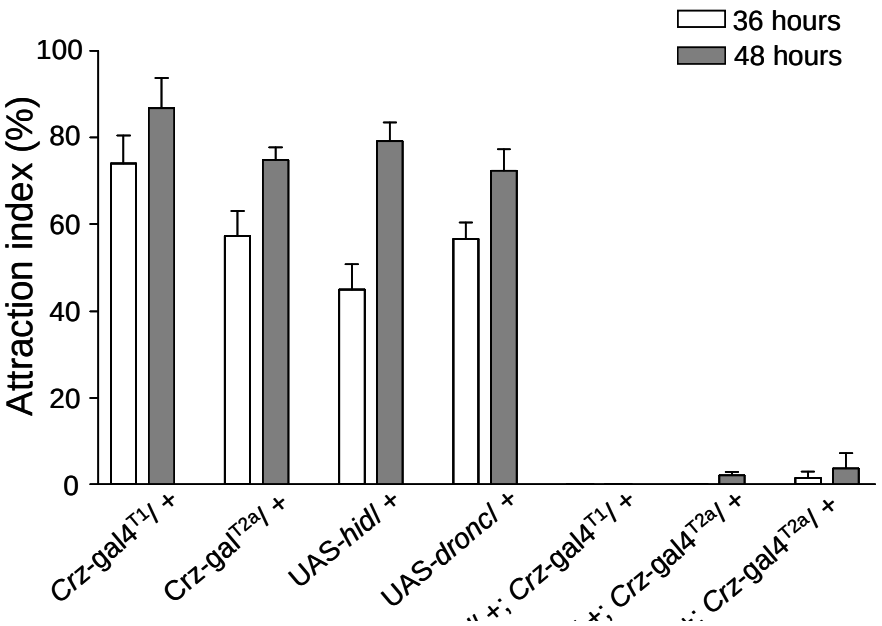


Fig. 5-6. Olfactory trap assay in Crz-CD flies using yeast paste as an attractant. The flies were allowed to find the odor source for 48 hours. CRZ expressing cell knocked out flies showed very severely less sensitive to find the yeast paste source than control groups. The females of *y w* and *Crz*-gal4 transgenic flies (*y w* background) were crossed with the males of *UAS-hid* (*y w* background) and *UAS-dronc* (*w* background). Each groups were tested at least 5 trials. The error bars indicate standard deviation. Open bar represents the percentage of entrapped flies 36 hour after the test and solid bar represents 48 hour after the test (n≥5, error bar=S. D.).

comparing with starvation. For example, flies started to entrap after 36 hours (data not shown). As expected, flies were entrapped in a time-dependent manner. They started to enter the trap at 24 h after flies were released into the arena (data not show). At 36 h, 55~70% of control flies were found in the yeast paste trap, but Crz-CD flies failed to find the odor source (Fig 5-6 white bar). At 48 h, more than 75% of control flies attracted to the odor source, while, very few Crz-CD flies did so (Fig 5-6 gray bar).

Based on this result, we wandered if ectopic expression or overexpression of *Crz* causes enhanced sensitivity to odor perception. To test this, we generated *UAS-Crz* transgenic flies to produce mature Crz in specific tissue. *UAS-Crz* flies were crossed with a pan-neuronal driver (*elav-gal4*) to express *Crz* in a whole CNS (Song et al., 2002). In the larval stage, some extra cells expressed *Crz* in brain and VNC regions (Fig 5-7 B, arrow and arrowhead), but not in all neurons (Fig 5-7 B). Alternatively we crossed *UAS-p35* with *Crz-gal4* driver. Previously, we demonstrated vCrz cells undergo programmed cell death during metamorphosis. The programmed cell death can be successfully blocked by expression of baculoviral anti-apoptotic protein *p35* expression in vCrz neurons (Choi et al., 2006). Unexpectedly, *Crz* over-expressed (hereafter we called Ox-Crz) flies showed hyposensitivity. At 36 h, Ox-Crz flies showed around 25% attraction index, compared to controls that showed more than 60% (Fig 5-7 A). At 48 h, Ox-Crz flies attracted into yeast around 40%, compared to more than 80% of controls (Fig 5-7 A). Ox-Crz flies are defective in odor perception at the levels in between wild-type and Crz-CD flies (Fig 5-6 and 5-7 A).

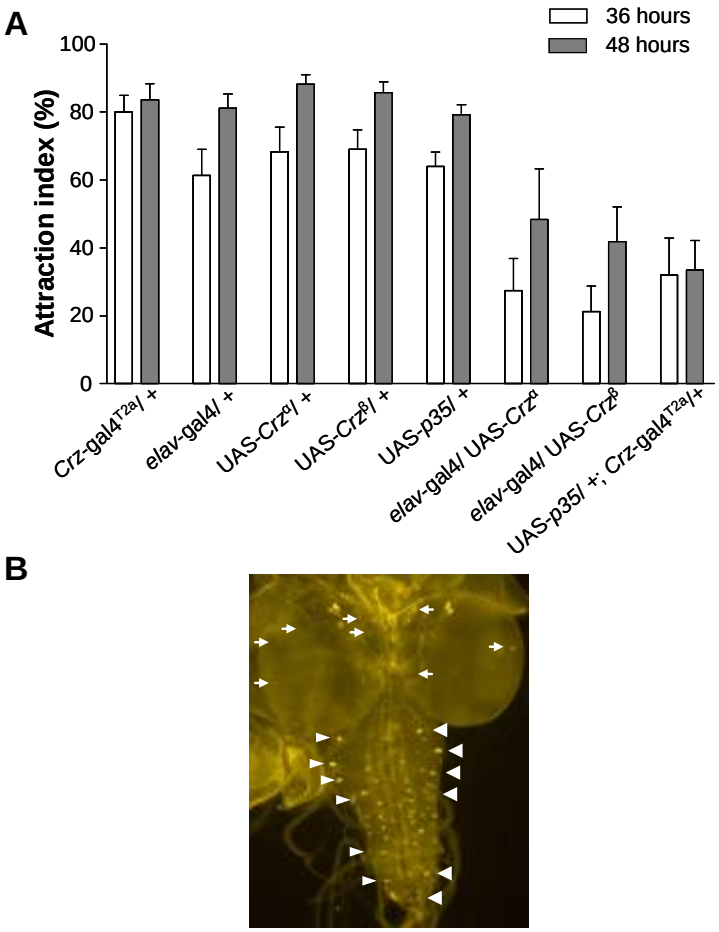


Fig. 5-7. Olfactory trap assay of Crz over-expression flies using yeast paste as an attractant. (A) The flies were allowed to find the odor source for 48 hours. The female of *y w*, *UAS-Crz* (*y w* background) and *Crz-gal4* (*y w* background) transgenic flies were crossed with pan-neuronal driver *elav-gal4* (*w* background) and baculoviral anti-apoptotic protein p35 respondent (*w* background). Crz over-expressing flies showed less sensitive to find the yeast paste source than did control groups, but more sensitive than did Crz-CD flies. Each group as tested at least 5 trials. The error bars indicate standard deviation. Open bar represents the percentage of entrapped flies 36 h after the test and solid bar represents 48 h after the test (n=5, error bar=S. D.). (B) *Crz* over-expression driven by *elav-gal4* pan-neuronal driver. Ectopic Crz expression neurons are indicated by arrow (in brain) and arrow head (in VNC).

Blocking *Crz* neuronal activity affects olfactory sensing

The *Crz* neurons co-expressed diuretic hormone receptors (Johnson et al., 2005). It suggests that *Crz* neurons are activated by other signal molecules such as diuretic hormone, and these activated neurons participated in odor perceptions. The defective chemical sensing phenotype in Crz-CD flies might reflect functions of the *Crz* neurons not *Crz* neuropeptide. Therefore we decided to block neuronal activity via several transgene expressions in *Crz* neuron. To do this, we expressed inactive protein kinase A regulating subunit (*PKA*^{inh}) that is deficient in cAMP binding. PKA consists of two regulatory subunits and two catalytic subunits. PKA^{inh} protein lacks cAMP binding, thereby overexpression of PKA^{inh} interfering with binding of normal regulatory subunits with catalytic subunits (Rodan et al., 2002). The other way of neuronal inactivation is to express of modified Shaker K^+ channel (called *EKO*) in *Crz* neurons. This channel has point mutations in transmembrane domain to block voltage-induced channel opening. When *EKO* is expressed in neurons, it blocks depolarization, thus inactivate neuronal functions (White et al., 2001). On the contrary to block the neuronal activity, *Crz* neurons enhance their activity through the expression of bacterial sodium channel (*NachBac*). This channel is activated by voltage and blocked by calcium channel blocker (Ren et al., 2001). The *NachBac* expression induces constitutive activation via delaying of depolarization (Luan et al., 2006).

Recipient flies (*UAS-PKA*^{inh}, *UAS-3X EKO* and *UAS-NachBac*) were crossed to the *Crz-gal4* driver. The males derived from each crossing were collected and performed olfactory trap assay. When we expressed *PKA*^{inh} and *EKO* in *Crz* neurons, flies showed defective olfactory sensing phenotype. At the level similar to Ox-Crz flies;

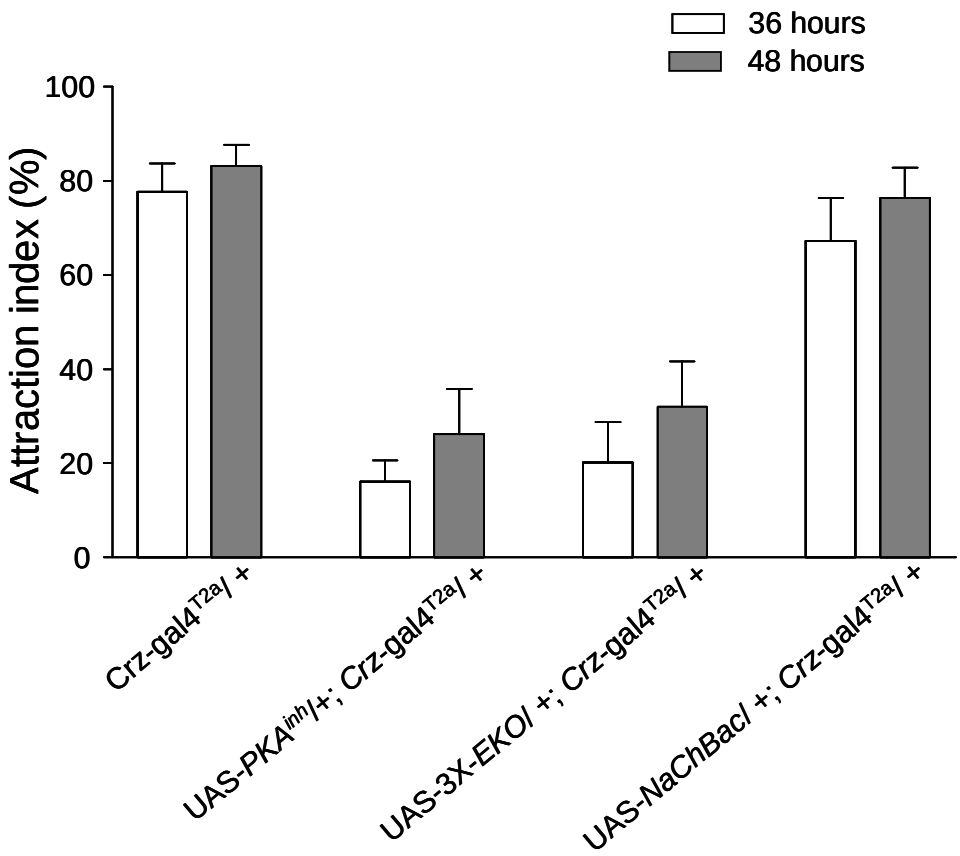


Fig. 5-8. Olfactory trap assay in the inactivation of *Crz* neuronal function impairs olfactory perception. The female of *y w*, and *Crz*-gal4 (*y w* background) transgenic flies were crossed with protein kinase A inhibitor (PKA^{inh}, *w* background) or shaker K^+ channel (EKO, *w* background) to inhibit *Crz* expressing neuronal function. Each group was tested at least 3 trials. The error bars indicate standard deviation. Open bar represents the percentage of entrapped flies 36 h after the test and solid bar 48 h after the test (n=5, error bar=S. D.).

for example, at 36 hours, ca. 20% flies entrapped into the yeast trap (Fig 5-8, White bar) and around 35% flies at 48 hours (Fig. 5-8, Gray bar). However, *NachBac* expression in *Crz* neuron did not show any difference, compared to controls (Fig 5-8).

CrzR-KD flies also showed defective chemosensory phenotype

As describe in Chapter IV, we generated CrzR-KD flies through *CrzR* RNAi induction (Fig. 4-12). The *UAS-CrzR*^{RNAi} flies were crossed to a universal driver *actin-gal4*, and the progeny were tested for their olfactory performance. As expected, CrzR-KD flies showed defective odor perception phenotype. At 36 h, ca. 40% of CrzR-KD flies found in the yeast trap. At 48 h, attraction index was increased to 60% (Fig. 5-9). We also tested putative *CrzR* mutant, *CrzR*^{minos} flies. In contrast to CrzR-KD, *CrzR*^{minos} flies showed some variation. We tested more than 12 different batches of flies; there of which showed results comparable to the controls (~80% at 48h), which 9 of which showed results similar to Crz-KD flies. Therefore, we could not make a conclusion whether *CrzR*^{minos} flies are defective in olfactory sensing (data not shown)

Crz-CD flies delay to find sources of single chemical

We wondered whether defective odor perception phenotype in Crz-CD flies is specific to yeast or single chemical. *Drosophila* attracted or repulsed different class of chemicals; acids, aldehydes, acetates and alcohols (McKenna et al., 1989; Woodard et al., 1989; Larsson et al., 2004). We used three different acids that are known to attract flies. When 1% propionic acid was used, Crz-CD flies showed delayed response. At 12 h, flies entrapped less than 20%, which were not different among genotypes. From 24 h,

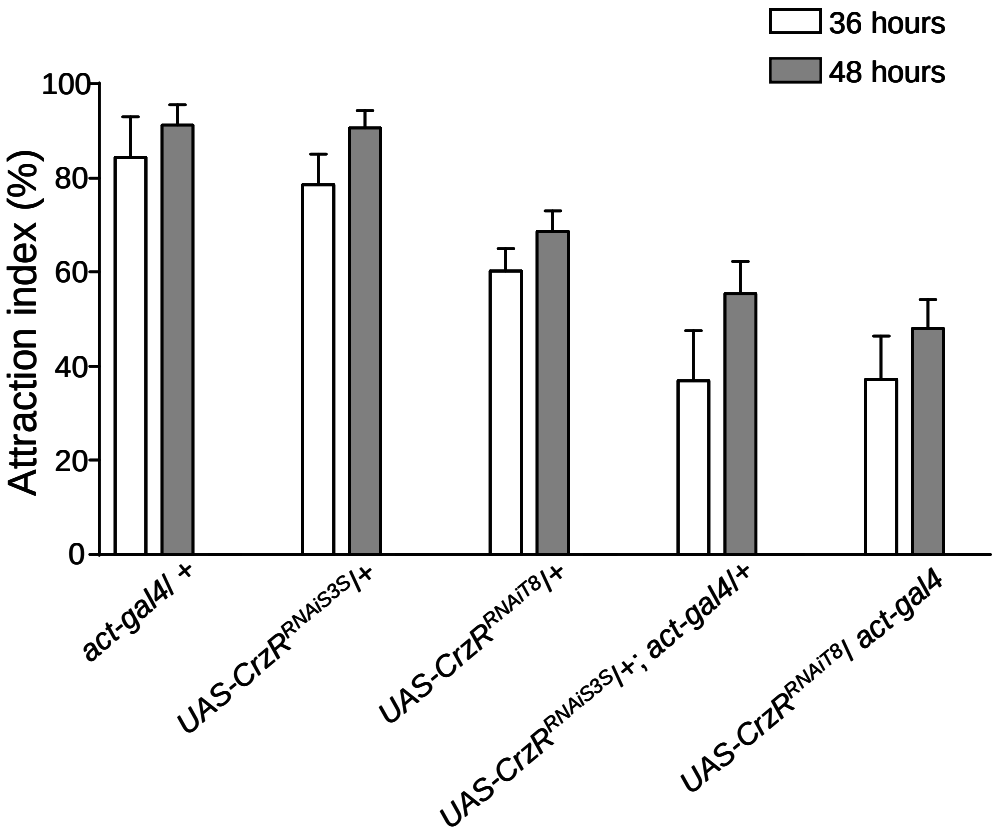


Fig. 5-9. Olfactory trap assay of *CrzR*-KD flies by CrzR-RNAi expression using yeast paste as an attractant. The female of *y w*, and *UAS-CrzR-RNAi* (*y w* background) transgenic flies were crossed to *actin-gal4* driver to knockdown the *CrzR*. The error bars indicate standard deviation.

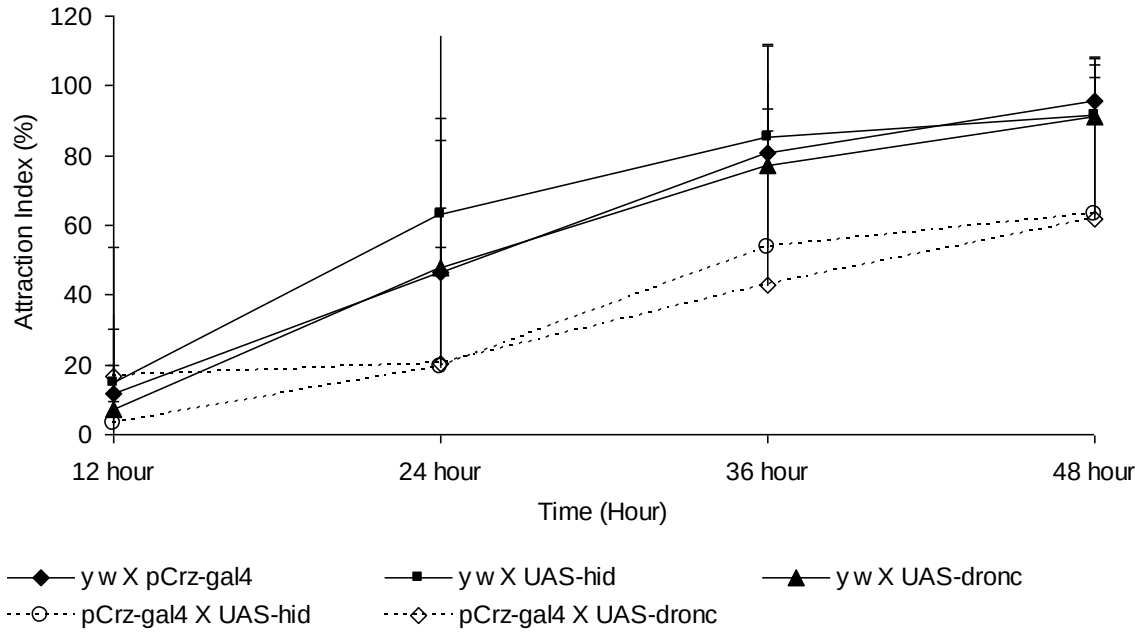
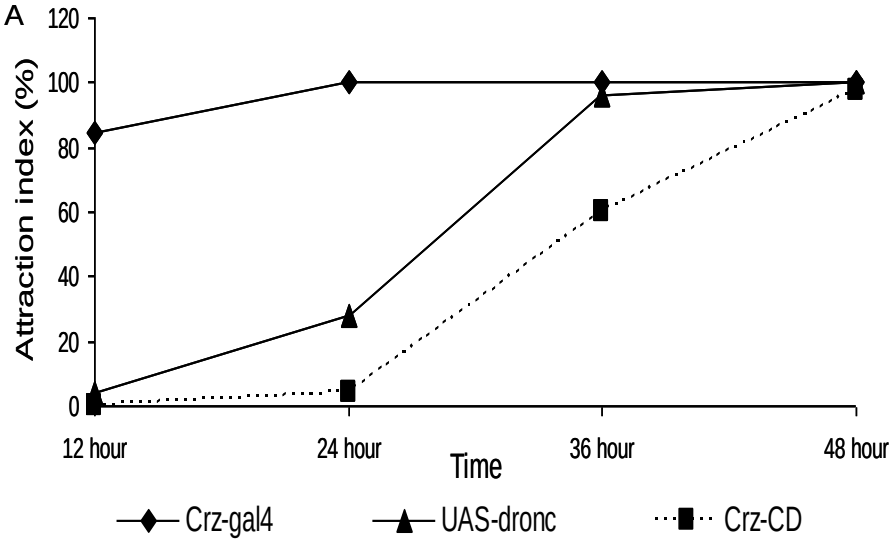


Fig. 5-10. Olfactory response to 1% propionic acid. Crz-CD flies (dashed lines) showed less response to 1% prooionic aicd (n=3, error bar=S. D.).



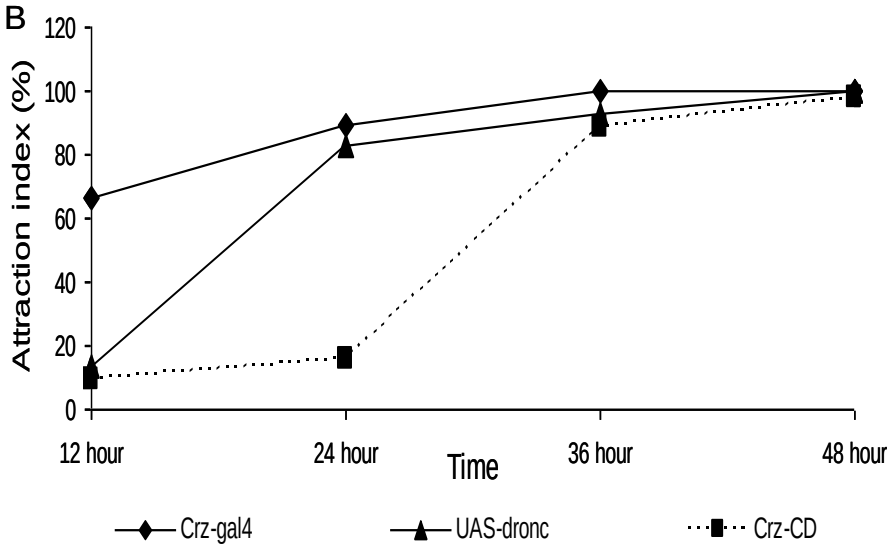


Fig. 5-11. Olfactory response to (A) 1%, and (B) 0.1% acetic acid. Crz-CD flies (dashed lines) showed delayed response to 1%, and 0.1% acetic aicd comparing control flies (solid lines; n=1).

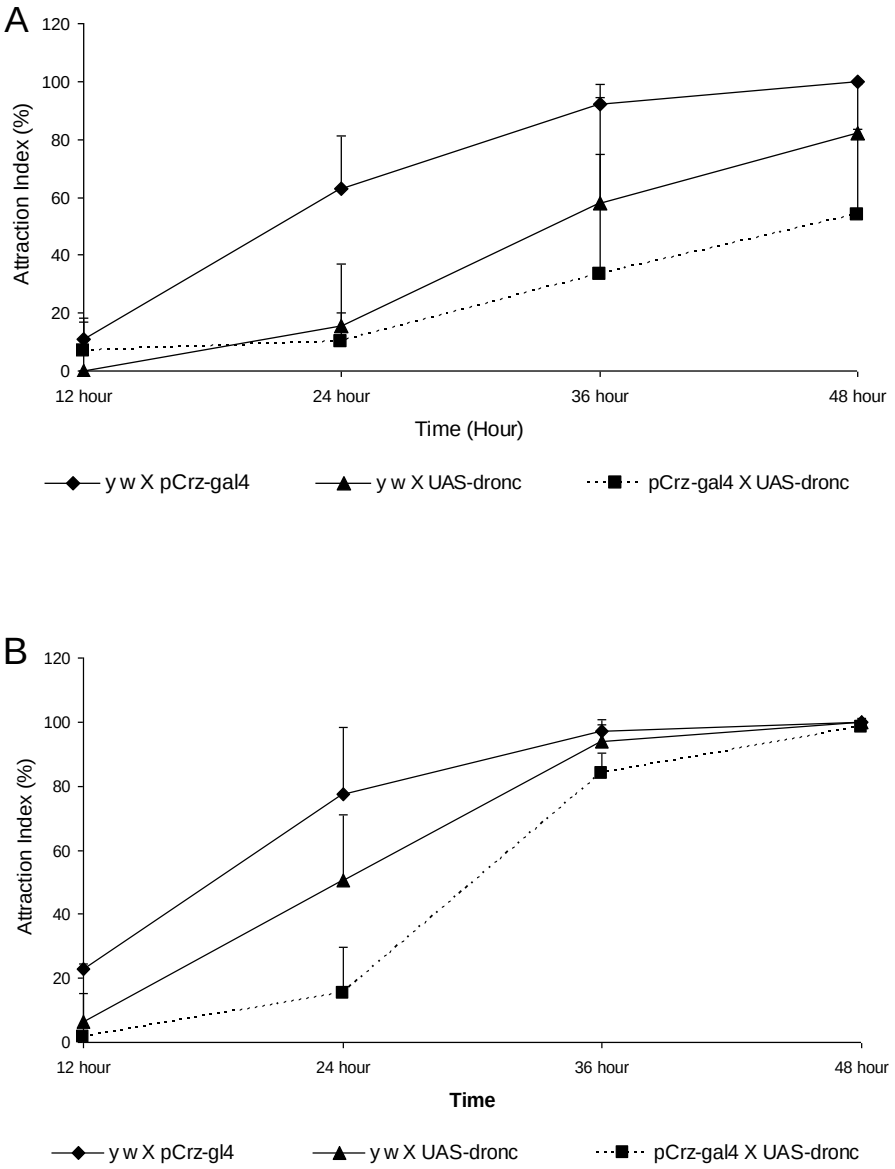


Fig. 5-12. Olfactory response to (A) 1%, and (B) 0.1% 2-phenylethanol. Crz-CD flies (dashed lines) showed less response to 1% 2-phenylethanol comparing control flies (solid lines; n=3, error bar=S. D.; A). However at a low concentration, Crz-CD flies showedless response at 24h, but did not show any significant difference at 36 and 48 h (B).

onward Crz-CD flies showed less degree of response, as 20% of Crz-CD flies entrapped, which 40% of control flies entrapped. At 48 h, attraction index of control flies reached almost 100%, but Crz-CD flies entrapped around 60% (Fig 5-10).

Using acetic acid as an attractant, we found similar results with propionic acid. At 1% concentration, ~60% of Crz-CD flies were entrapped at 36 hours, while all control flies were entrapped at the same time point (Fig 5-11, A). At a low concentration (0.1%), fly responses faster than 1% concentration, but delayed response of Crz-CD flies maintained. In control, attraction index reached almost 100% at 24 h, but comparable attraction index for Crz-CD flies was obtained at 36 h (Fig 5-11, B). Interestingly, *Crz-gal4*/+ flies showed very high attraction index (A. I.) even 12h after the assay.

We tested other group of chemicals, alcohols, as an attractant. Crz-CD flies are less sensitive to 1% phenylethanol; at 36 h, less than 40% Crz-CD flies entrapped comparing to controls entrapped more than 60%. At 48 h, Crz-CD flies showed 60% A. I., while control flies 80% A. I. (Fig 5-12, A). At a low concentration (0.1%), Crz-CD flies showed lower A. I. at 24 h, but other time points, flies did not show any differences (Fig 5-12, B). Using 0.1% n-s butanol as an attractant, Crz-CD showed lower A. I. at 24 h and 36 h (Fig 5-13, A); however, at a lower concentration (0.025%), Crz-CD flies showed lower A. I. only at 24 h (Fig 5-13, B).

In case of 0.5% ethyl acetate, Crz-CD flies entrapped lower number than control flies. A. I. of controls reached almost 100% at 36 h, but that of Crz-CD flies, reached 100% even at 48 h (Fig. 5-14, A). At a lower concentration (0.05% ethyl acetate), fly response was much faster. The A. I. reached 100% less than in 24 h in control flies, while A. I. of Crz-CD flies at the same time point, reached around 50%, however, it

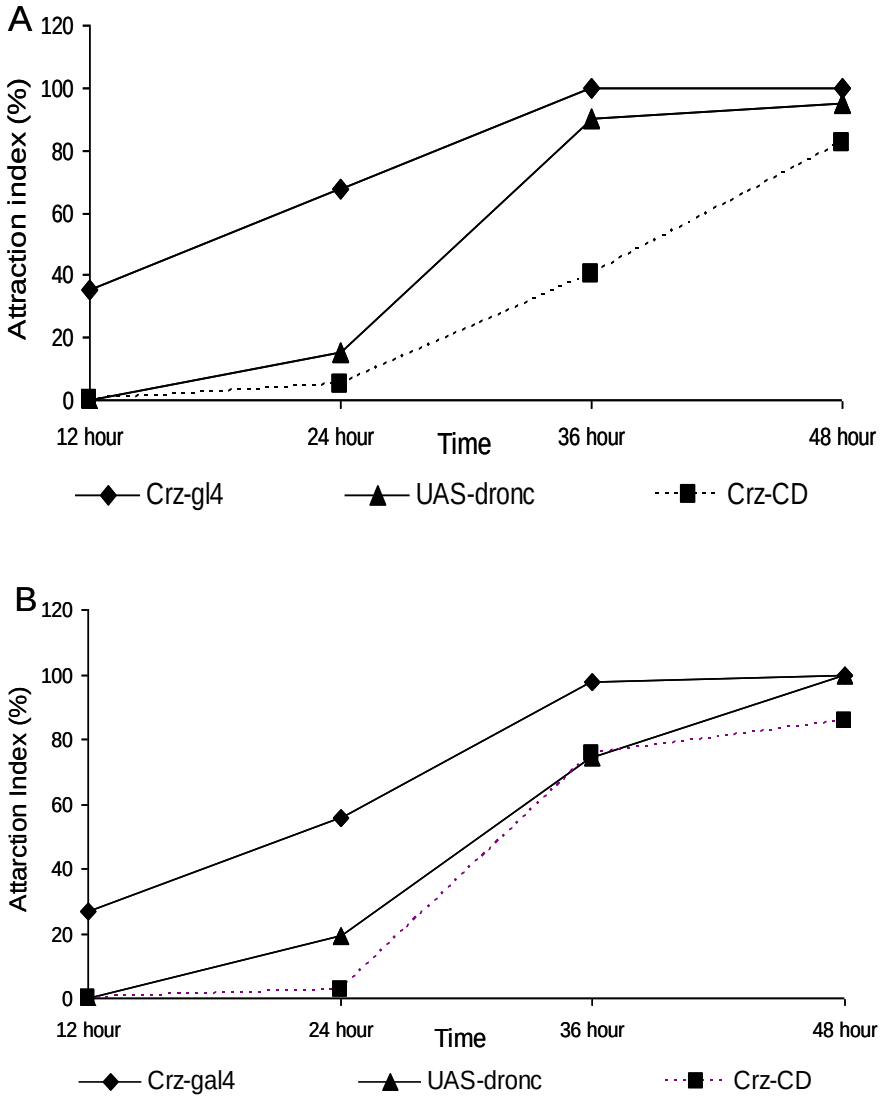


Fig. 5-13. Olfactory response to (A) 0.1%, and (B) 0.025% n-butanol. Crz-CD flies (dashed lines) showed delayed response to 0.1% n-butanol compared to control flies (solid line; A). However at 0.025% concentration, Crz-CD flies showedless response at 24h, but did not show any significant difference at 36 and 48 h (B; n=1).

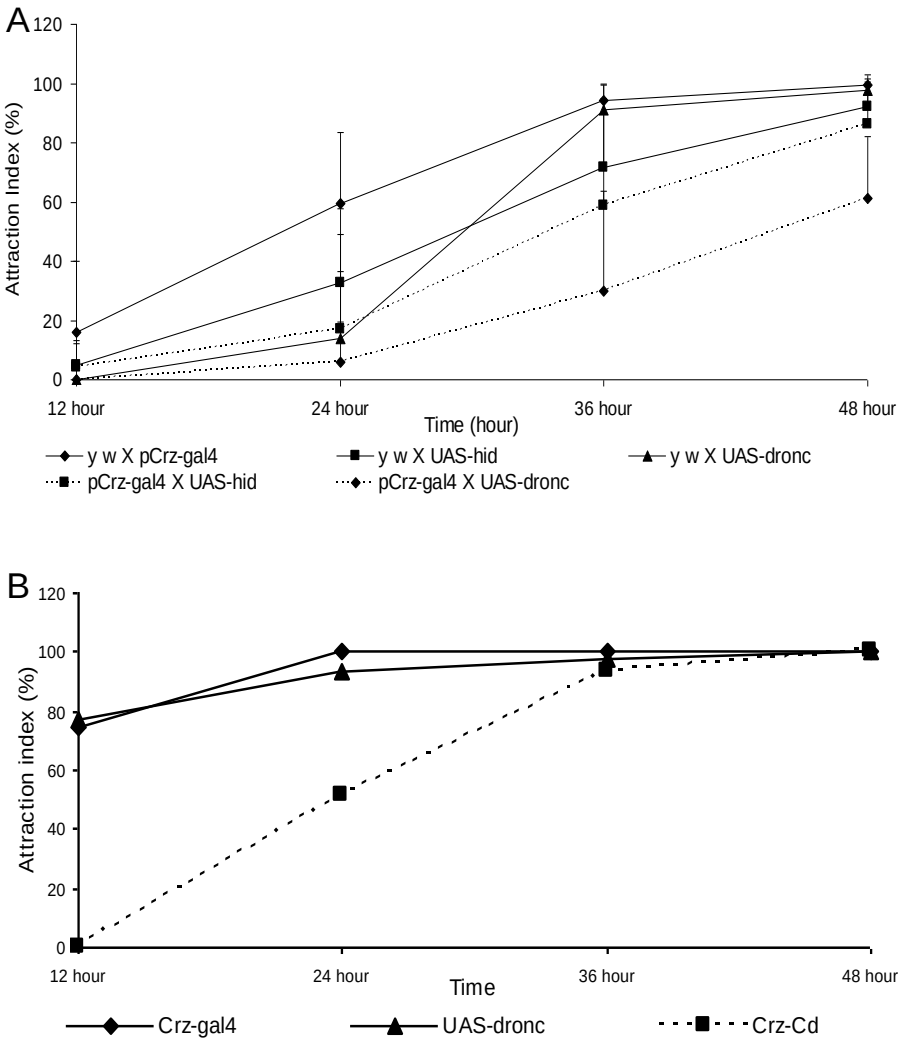


Fig. 5-14. Olfactory response to (A) 0.5%, and (B) 0.05% ethylacetate. Crz-CD flies (dashed lines) showed less response to 0.5% ethylacetate compared to control flies (solid line; n=3, error bar=S. D.; A). However at a low concentration, flies showed delayed response at 12 h and 24 h (B; n=1).

reached almost 100% at 36 h (Fig. 5-14, B).

We also tested ketone groups such as acetone and n-butanone. With 1% acetone, Crz-CD flies showed less sensitive response. At 36 h, more than 95% of control flies found source of acetone, but Crz-CD flies entrapped less than 70%. At 48 h, Crz-CD flies still entrapped under 80% in the acetone trap (Fig. 5-15, A). However, this less sensitive response disappeared in lower concentration. At 0.1% acetone, Crz-CD flies did not show any significant difference comparing to control flies (Fig. 5-15, B). Another ketone group chemical, n-butanone, was showed less sensitive response. However, the level of sensitivity is severe than acetone. With 1% n-butanone, more than 90% of control flies entrapped at 36 h, but Crz-CD flies showed less than 40% in the trap. Even at 48 h, at least 50% of Crz-CD flies did not find odor source (Fig 5-16).

***Crz* does not involve *Drosophila* life-span**

Recently, a relationship between life-span and odor perception was reported. Mutation of broadly expressed olfactory receptor *Or83b* showed severe defect in olfactory responses, changed adult metabolism, enhanced stress resistance and elongated life spans (Larsson et al., 2004; Benton, 2006; Libert et al., 2007). *Or83b* mutant flies live about 30% longer than wild-types (Libert et al., 2007). It indicates olfaction affects adult physiology and longevity in *Drosophila*, possibly via recognition of nutrient source and regulation of aging related genes. In this respect, we were interest in whether Crz-CD affects the life span or not. To accelerate fly's metabolism, flies

were raised at 29℃. As a result, we found that *Crz* does not affect fly's life span. Crz-CD, CrzR-KD and *CrzR*^{minos} did not show any differences in their life span compared to

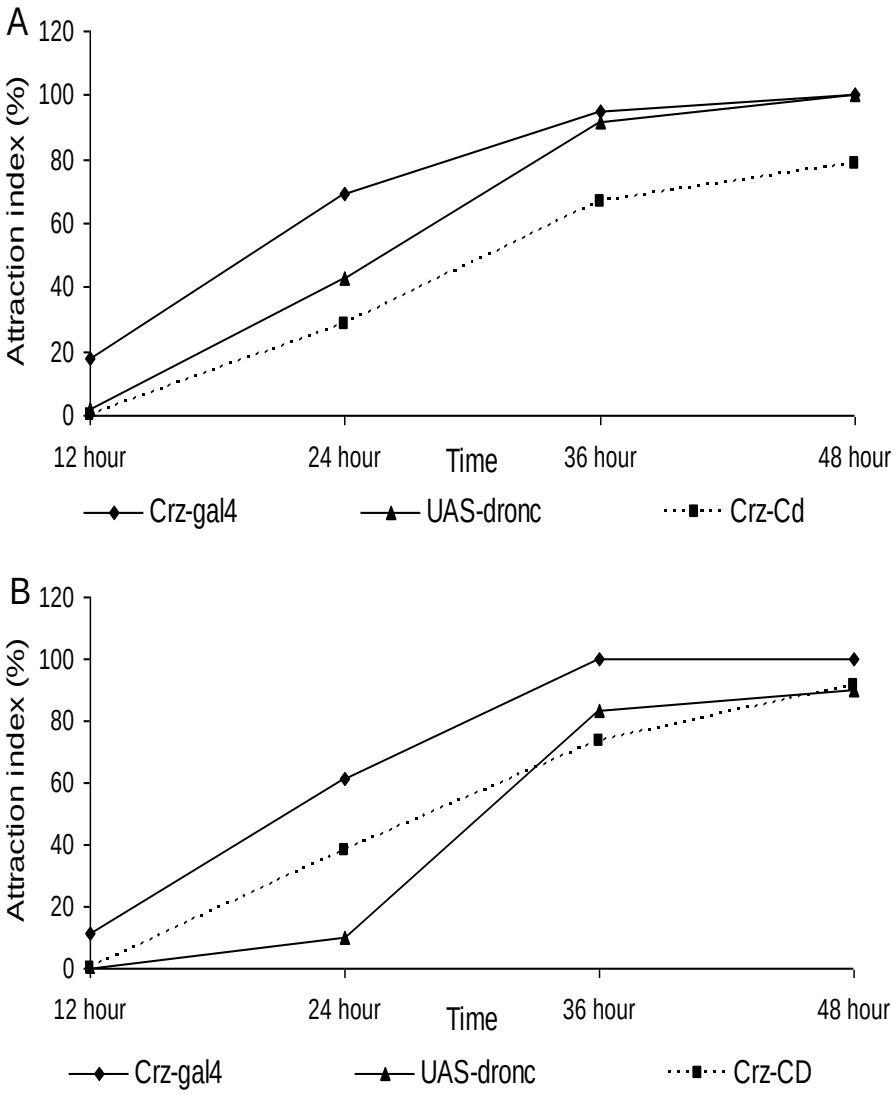


Fig. 5-15. Olfactory response to (A) 1%, and (B) 0.1% acetone. Crz-CD flies (dashed lines) showed delayed response to 1% acetone, however at 0.1% concentration, flies does not show any significant difference (B; n=1).

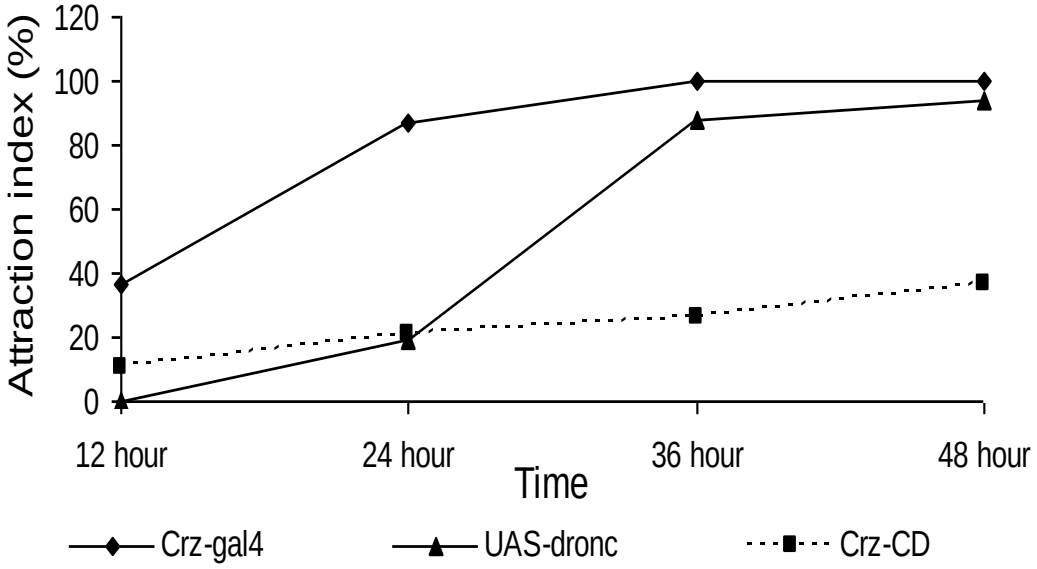


Fig. 5-16. Olfactory response to 1% n-butanone. Crz-CD flies (dashed lines) showed significantly less sensitive response to 1% n-butanol compared to control flies (solid line, n=1).

control flies (Fig 5-17). To verify *Crz* specific function, we added two different neuropeptide expressing neuron ablated flies, npf and Akh. The npf and Akh cell ablated flies did not show any significant differences from Crz-CD, CrzR-KD and *CrzR*^{minos} flies (data not shown).

Crz does not affect number of olfactory sensilla

Crz injection in locust was reported to decrease the total number of antennal sensilla, especially basiconic and coleoconic sensilla (Maeno et al., 2004). Crz-CD, Ox-Crz, CrzR-KD and *CrzR*^{minos} defect in olfactory perception, could be due to the reduced number of sensilla in the antenna. In *Drosophila*, most of olfactory sensilla exist on the 3rd antennal segment (Fig. 5-18). *Drosophila* has three different sensilla types; trichoid, basiconic and coleconic (Hallem and Carlson, 2004, 2004). To determine whether *Crz* participates in the development of olfactory sensilla, we counted trichoid number on the 3rd antennal segment. Crz-CD, CrzR-KD and *CrzR*^{minos} did not show difference in trichoid number. Our finding indicates that *Crz* is important for olfactory response; however, Crz does not involve in the structural development of the chemosensory machinery.

V. Discussion

This report is concerned with a novel function of neuropeptide *Crz* in chemical perception. Chemosensory is the basal instinct to maintain life, especially, finding food sources and mating partner, and avoiding harmful materials (Alcorta and Rubio, 1989; Helfand and Carlson, 1989; McKenna et al., 1989; Woodard et al., 1989). Fundamental

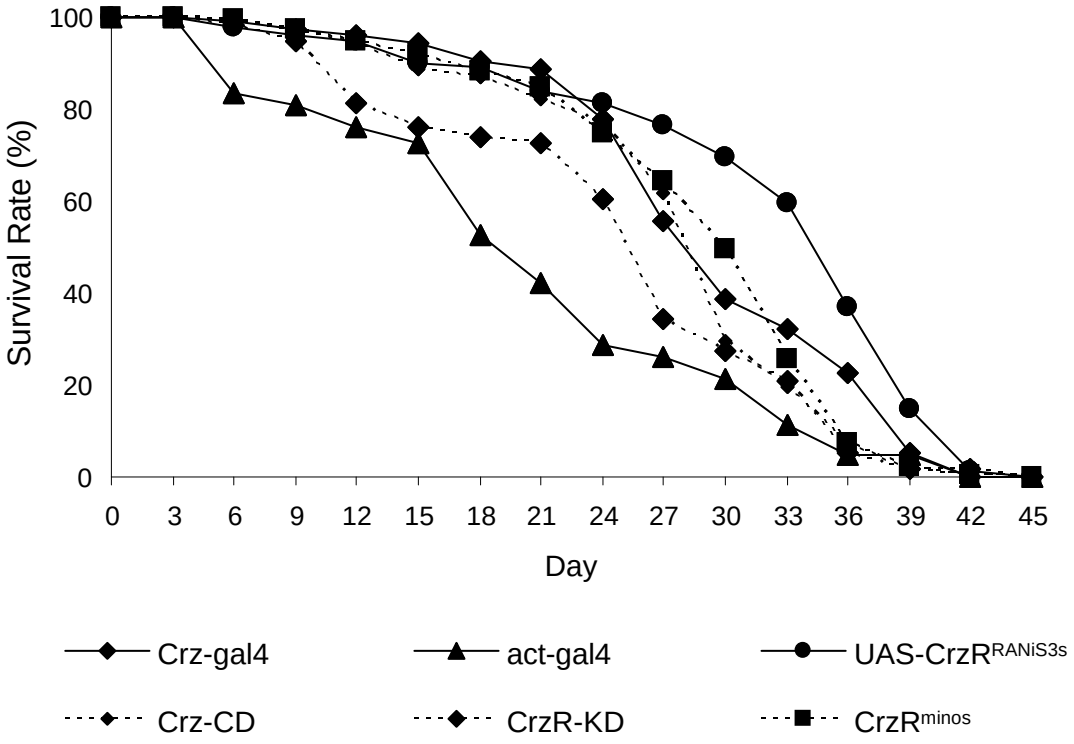


Fig. 5-17. Longevity test. Crz-CD, CrzR-KD, and *CrzR*^{minos} flies (dashed line) did not show any significant differences comparing to control flies (solid line).

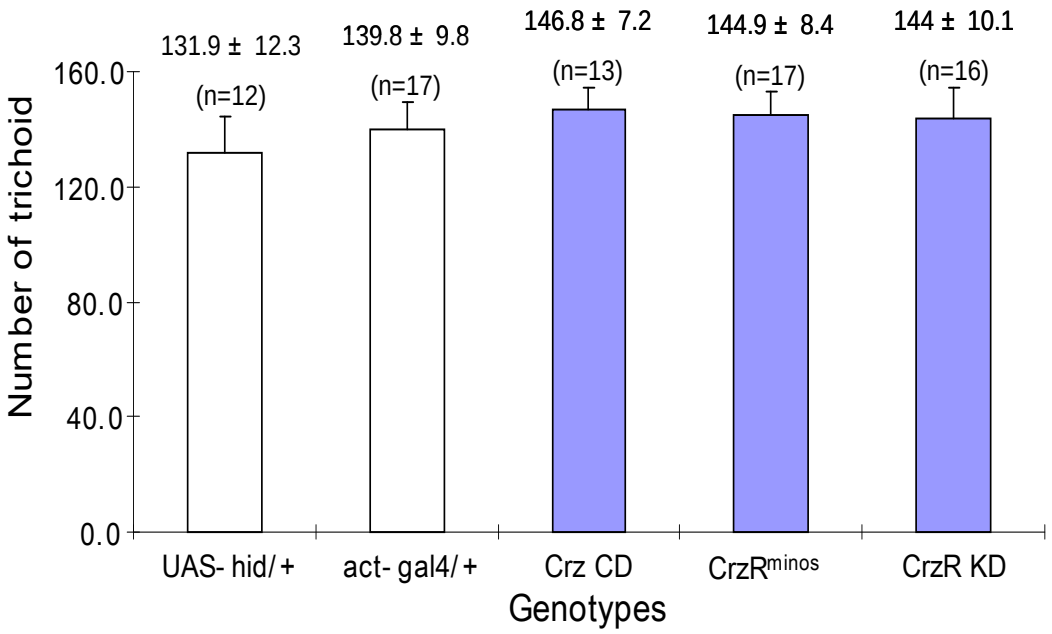


Fig. 5-18. Number of trichoid sensilla on the 3rd antennal segment. The number of Crz-CD, CrzR-KD, and *CrzR*^{minos} flies (solid bars) are not significantly different from control flies (open bars, n ≥ 12).

chemoperception show similarity in many aspects between mammals and fruit fly; even the topology of olfactory receptors and combinations are different (Alcorta Alcorta and Rubio, 1989; Bozza et al., 2002; Hallem et al., 2004). We characterized severe defect of chemical sensing in Crz-CD flies.

Crz involved in odor recognition

We revealed that neuropeptide *Crz* signaling participates in the detection of the location of chemical sources. Without Crz, flies does not find source of yeast paste (Fig. 5-6). We assumed that overexpression of *Crz* causes hypersensitive to odor. On the contrary, when we express *Crz* ectopically using *UAS-gal4* system (Fig 5-7, B), fly showed lower or defect odor perception. These results suggest that *Crz* regulated very tightly and threshold is very narrow; too much or too small amount of Crz affects appropriate response to the chemical stimuli.

Crz-CD flies did not find the source of yeast paste. Live yeast produces a number of volatile chemicals, including alcohols, acids, ketones, and aldehydes (Antonelli et al., 1999). To find core chemicals for olfactory defect of Crz-CD flies, we tested several single chemicals as an attractant; the flies showed delayed or modest responses (Fig 5-10 through 5-16). Therefore we assume that defective chemical sensing with yeast paste come synergistic effect from several single chemicals. These days, many researchers do electroantennogram (EAG) to check the neuronal activities against single chemicals (Larsson et al., 2004; Hallem and Carlson, 2006; Benton et al., 2009; Lei et al., 2009; Riffell et al., 2009). We need to further perform EAG or

extracellular recordings in a single sensillum for finding individual sensilla activity in Crz-CD, CrzR-KD and *CrzR*^{minos} flies.

***Crz* does not involve *Drosophila* life span**

Recently, a new link between life span and odor perception was reported. The nutrient restricted flies increased their life span via reducing lipid oxidative damage or changing in age-dependent gene expressions (Pletcher and Stumpf, 2002; Bross et al., 2005; Zheng et al., 2005). More directly, broadly expressed olfactory receptor *Or83b* mutant flies live 30% longer than wild-type flies. Although feeding and olfaction have interaction, *Or83b* mutant does not have a direct connection with food uptake. This result indicates that olfactory sensing has diet-independent pathway to extend animal's life span (Libert et al., 2007).

Crz-CD, Ox-Crz, and CrzR-KD flies are defective in olfactory sensing. Therefore, it is possible that fly neuropeptide *Crz* controls fly longevity. However, they showed normal life span. This finding leads to the possibility that Crz mediated olfactory detect is not associated with olfactory receptors. Two models are suggested. One is *Crz* participates in control of activity of projection neuron in the glomeruli. Olfactory sensing mechanisms are not well understood. When ORs bind odorant, activated signals are transmitted to glomeruli on the antennal lobe and then projection neurons are participated in the signal transmission to the mushroom body and lateral horn (Hallem and Carlson, 2004). *Crz* might modulate these projection neuronal activities. Without or too much *Crz*, projection neurons on the antennal lobe decreases or loose their activities, leading to suboptimal chemical perception. Another possibility is that *Crz* is

related to new olfactory sensing mechanism. Recently, novel olfactory receptor family, sharing iGluR domain, was identified. These iGluR-like receptors are mainly existed on coleoconic sensilla, arista and sacculus, whereas, ORs exist mainly on trichoid and basiconic sensilla (Vosshall and Stocker, 2007; Benton et al., 2009). Crz-CD and CrzR-KD flies did not change the number of trichoid sensilla (Table 5-1). This finding might indicate that *Crz* involves iGluR-like receptor mediated olfactory sensing. We need to further investigate to find a relationship between Crz and iGluR-like olfactory receptors. It will be interesting to check the number and activity of coleoconic sensilla in Crz-CD, CrzR-KD and $CrzR^{minos}$ flies.

Chapter Six

Function of *Corazonin* in Circadian Rhythms

I. Abstract

Circadian rhythms in animals are governed by endogenous neurons. *Crz* neurons are located in the vicinity of nerve terminals originated from circadian pacemaker Pdf-expressing neurons. This observation indicates *Crz* neurons might be part of the circadian circuit. However, Crz-CD, Crz over-expression and CrzR-KD flies show normal circadian locomotor activity rhythm, suggesting that *Crz* neurons are not involved in this type of biological rhythms in *Drosophila*.

II. Introduction

Organisms on earth have adapted to environmental fluctuation, such as daily and seasonal changes in light and dark, temperature, humidity and other physical parameters. Although almost all parameter reflects passively, organisms have biological clocks, which permit them to anticipate and prepare daily change of environmental conditions. This biological clocks lead organisms' time specific activities to certain phases in the 24-h cycles, such as metabolic and physiological oscillations of their corresponding environmental times. In the absence of environmental rhythmic fluctuations, endogenous clocks are maintained ca. 24-h period, which is called circadian rhythms.

The timekeeping mechanisms are synchronized by environment. One of the most important cues of biological clocks in nature is the daily light and dark cycles, which is recognized by specialized photoreceptors.

During past few decades, discoveries of circadian clocks genes and mechanisms were greatly advanced. The core mechanism of circadian clock is a molecular feedback loop in which transcriptional activation of clock genes is repressed by proteins encoded by same genes. In *Drosophila*, clock-related genes are revealed to be main components in central circadian rhythms, which are known: *period* (*per*), *timeless* (*tim*), *Clock* (*Clk*), *cycle* (*cyc*), *vrille* (*vri*), *double-time* (*dbt*), *cryptochrome*, *casein kinase 1*, and *shaggy* (*sgg*). The proteins, CLK and CYC, belongs to bHLH-PAS transcription factors (bHLH stands for basic helix-loop-helix domain and PAS stands for PER-ARNT-SIM, which are PAS protein family, Crews and Fan, 1999) and form a heterodimer to bind to specific recognition sequences within the promoters of the *per*, *tim*, and *vri* genes to activate their transcription. During early morning, CRYPTOCHROME (CRY) protein is activated by light and inhibited accumulation of TIM-PER heterodimer proteins via binding to TIM, while eventually leads to TIM degradation. Early evening, *per* and *tim* transcripts reach the highest level as CLK-CYC heterodimer activates both genes (Marrus et al., 1996; So and Rosbash, 1997). Phosphorylation of PER by DBT kinase causes its degradation (Price et al., 1998; Rothenfluh et al., 2000). Then cytosolic accumulation of TIM results in binding to PER and protects PER from degradation. The stabilized PER-TIM heterodimer enters the nucleus and inhibits *per* and *tim* transcript production via binding to CLK-CYC heterodimer, which induces the loss of the DNA-binding ability (Lee et al., 2001). TIM is gradually phosphorylated by another kinase

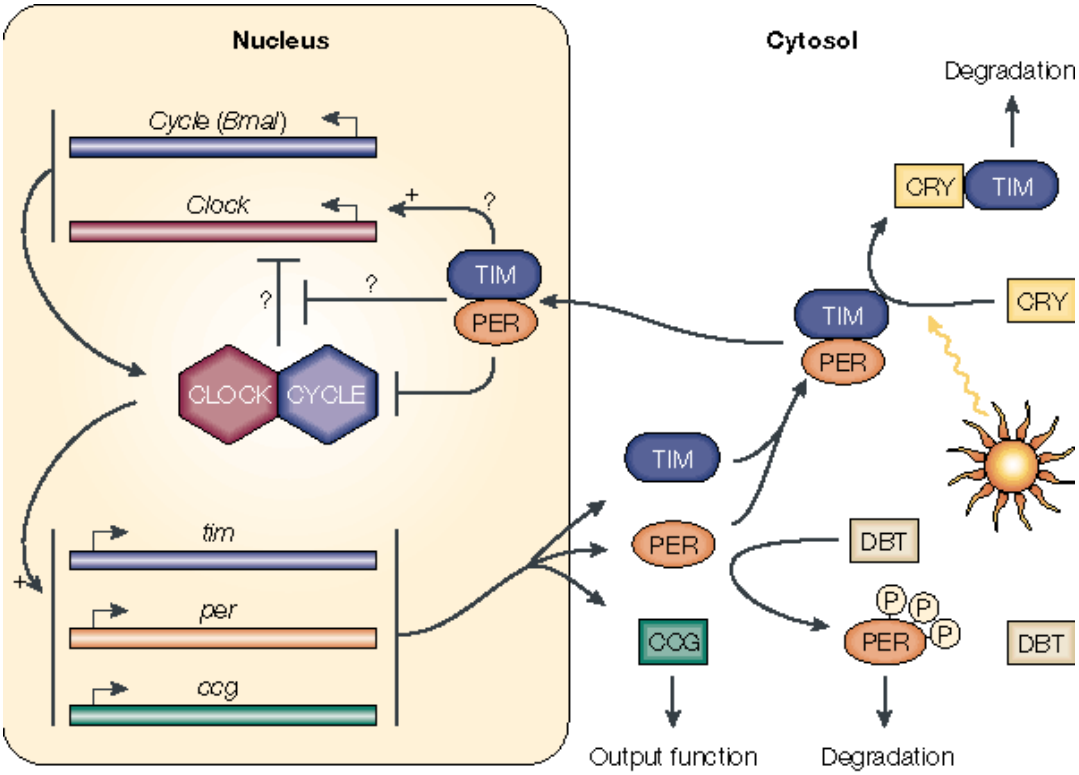


Fig. 6-1. Molecular feedback loop of clock genes. A negative autoregulatory loop of PER and TIM proteins on their own genes (through inhibition of the transcriptional activator CLOCK–CYCLE), and a positive regulation of clock genes on the expression of CLOCK. CRY and DBT proteins regulate the stability of the TIM and PER proteins. CLOCK–CYCLE also regulates clock-controlled genes (*ccg*). Figure from Cermakian and Sassone-Corsi, 2000

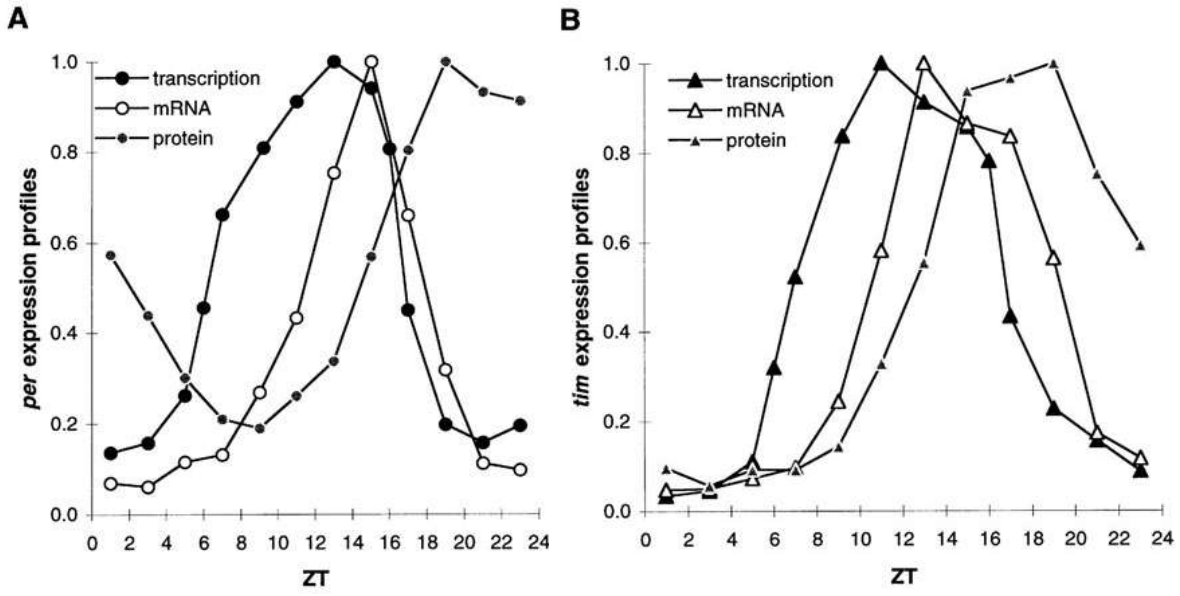


Fig. 6-2. Transcription, mRNA and protein oscillation of *per* (A) and *tim* (B). Protein profiles showed delay of transcription and mRNA profiles. Figure from So and Rosbash, 1997. ZT, zeitgeber (ZT0, light on; ZT12, light off).

SGG. This phosphorylation promotes the nuclear entry of the PER-TIM dimer (Martinek et al., 2001). Shortly after entry to nucleus, TIM protein degradation is induced probably by SGG and other unidentified tyrosine-kinases (Naidoo et al., 1999; Martinek et al., 2001). PER represses the CLK-CYC heterodimer until its degradation occurs via PER phosphorylatoin by DBT kinase (Rothenfluh et al., 2000). This degradation allows new round of *per* and *tim* transcription in the late morning (Fig. 6-1 and Fig. 6-2).

The feedback loop of oscillation is strengthened by another component VRI, which rhythmically represses the transcription of the Clk gene (Glossop et al., 1999). VRI includes a member of basic-Zipper (bZip) transcription factors, and regulated by CLK-CYC heterodimer in a manner similar to TIM and PER transcription factors (Fig. 6-1). In other word, *vri* gene is regulated by CLK-CYC and repressed by PER-TIM (Blau and Young, 1999). Therefore, the highest level of VRI protein is reached at the night and lowest at the end of the day.

As previously reported, *Drosophila* clock gene expressing neurons are well studied. Almost 150 circadian clock neurons are subgrouped according to their positions in brain, among these, a group of small ventro-lateral neurons (s-LN_v's) is particularly important for circadian locomotor rhythms, and the neuropeptide Pdf is an essential clock output factor that delivers clock information from this pace makers (Fig. 6-3; Renn Renn et al., 1999; Park et al., 2000; Schwartz, 2004; Helfrich-Forster, 2005). Interestingly, the s-LNv neuron project dorsally to the posterior protocereblum and their terminals arborize around *Crz* neurons (Choi et al., 2005). This result suggests that Crz may involve circadian rhythmic output regulations. Here, we tested the relationship between *Crz* and circadian rhythmic behavior.

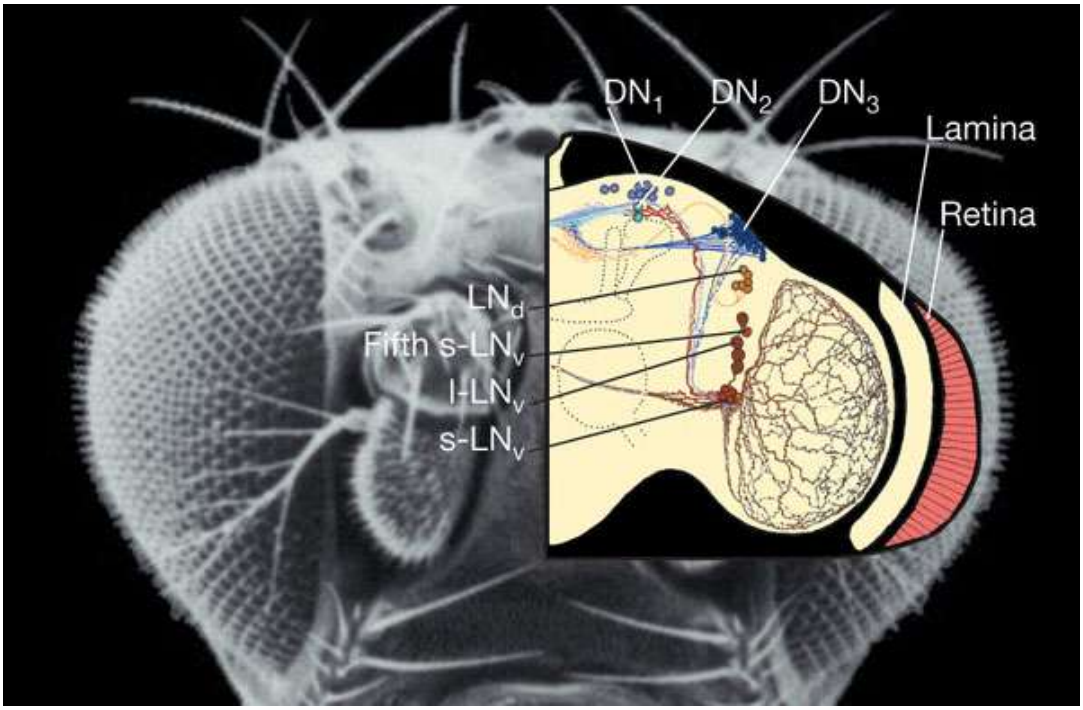


Fig. 6-3. Clock neurons in the brain of adult *D. melanogaster*. Each hemi brain has three groups of dorsal neurons (DN_1, DN_2, and DN_3) and two groups of lateral neurons, one dorsolateral (LN_d) and the other ventrolateral (LN_v: either large, l, or small, s). The fifth small LN_v cell is different from the other LN_v neurons in that it does not express the neuropeptide Pdf. Figure modified from Schwartz, 2004.

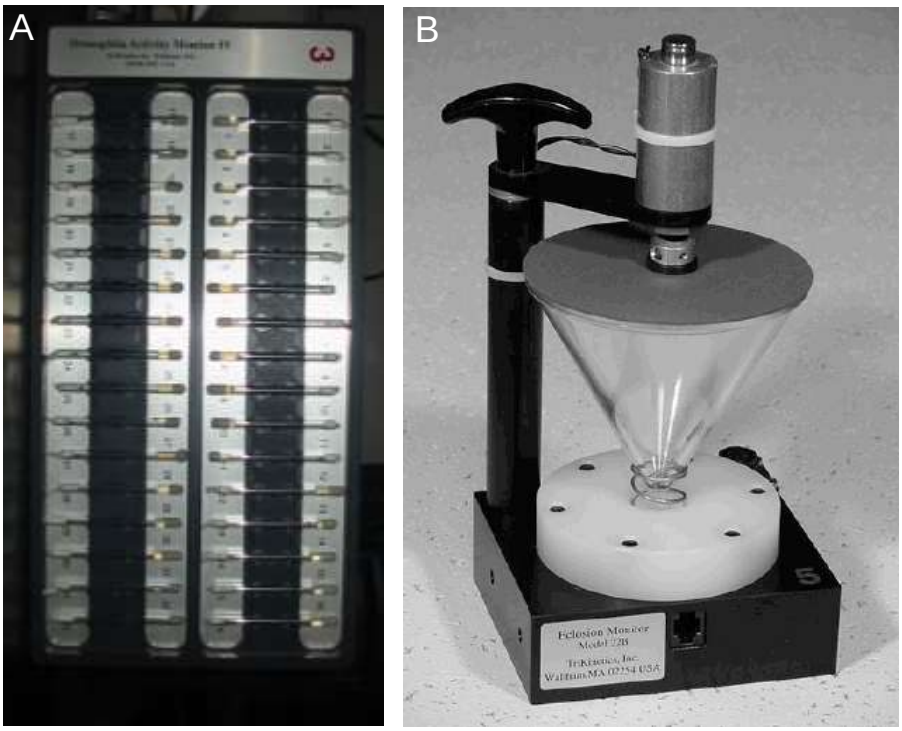


Fig. 6-4. Fly circadian behavior monitoring systems. (A) Drosophila activity monitor. (B) Drosophila eclosion monitor (Trikinetics).

III. Materials and Methods

Circadian behavioral rhythms

Flies were entrained for 3-4 days to 12-h light: 12-h dark cycles (LD) and then preceded into constant darkness conditions (DD). Locomotor activity of individual flies was monitored using *Drosophila* activity monitors (Fig. 6-4, A; Trikinetics). Data analysis was done with ClockLab software (Actimetircs).

Eclosion rhythm assay

Larvae were raised in 12-h light: 12-h dark cycles (LD conditions) at 25 ℃. Hundreds of pupae were collected and transferred to *Drosophila* eclosion monitors (Fig. 6-4, B; Trikinetics). The test was performed in constant darkness (DD conditions). Every 30 min, the solenoid tapping pushes down the disk/ funnel stack to count eclosed flies. Data analysis was done with Clocklab software.

Results

Crz does not affect fly biological clock

Fly locomotor behavior is one of the best characterized traits for biological clock (Konopka and Benzer, 1971; Hamblen et al., 1986). To understand whether Crz involves *Drosophila* circadian rhythms, we measured circadian locomotor activity rhythm.

Wild-type flies display bimodal activities under LD cycles, with gradual increase of locomotion prior to light on and light off. Under DD conditions, this bimodal activity

maintains more than a week (Fig. 6-5 A-B; Renn et al., 1999). Interestingly, under LD conditions, Crz-CD flies showed quiescent activity all day, startling activity followed

Table 6-1. Summary of the crossing for circadian behavior assay

♀♀	♂♂
y w	*Crz-gal4*^{T2a}
y w	*UAS-hid/ CyO, y*$^{+}$
UAS-hid/ CyO, y^{+}	*Crz-gal4*^{T2a}
UAS-p35	*Crz-gal4*^{T2a}
Pdf-gal4^{J}	*UAS-Crz*$^{\alpha}$
tim-gal4^{86}	*UAS-Crz*$^{\alpha}$
y w	*y w;; act-gal4/TM6C, Tb, Sb*
y w	*y w;; UAS-CrzR*^{RNAiS3S}
y w;; UAS-CrzR^{RNAiS3S}	*actin-gal4/TM6C, Tb, Sb*
CrzR^{minos}	
y w	*y w;; Crz-gal4*^{S2b}*; Pdf*^{01}
y w	*y w;; UAS-rpr; Pdf*^{01}
y w;; Crz-gal4^{S2b}*; Pdf*^{01}	*y w;; UAS-rpr; Pdf*^{01}

immediately after light on, and they started to move around 2 h before light off. Under DD conditions, Crz-CD flies disappeared morning movement, but maintained evening anticipation and rhythmicity (Fig. 6-5 E, F). This quiescent activity in Crz-CD flies might come from genetic background, as *UAS-hid*/ + heterozygous flies showed similar behavioral pattern to Crz-CD flies (Fig. 6-5 C, D).

Next, we examined the rhythmic behavior using Crz overexpression flies via genetic manipulation (summarized in Table 6-1). As previously mentioned, vCrz cells were protected from programmed cell death by anti-apoptotic protein p35 expression. Although, animals display very low or not active during day time, Ox-Crz flies via p35 expression in *Crz* neurons, maintain normal circadian behavior (Fig. 6-6 A, B).

Alternative way, we expressed *Crz* in clock neurons using *tim-gal4* and *Pdf-gal4* driver. When we expressed *Crz* in *pdf* neurons, flies showed bimodal behavior under LD conditions, and maintained their rhythmicity in DD conditions (Fig. 6-6 C, D). In contrast to Ox-Crz in *pdf* neurons, Ox-Crz in *tim* neurons showed abnormal behavior. Under LD conditions, flies moved constantly during night time, and locomotor activity displayed relatively weak rhythmicity with longer period (τ = 24.8 hr; Fig 6-6 E, F; summarized in Table 6-2).

We also tested whether CrzR-KD and $CrzR^{minos}$ flies show abnormal circadian rhythmic behavior. As shown for Crz-CD flies, CrzR-KD and $CrzR^{minos}$ flies did not show severe abnormality (Fig. 6-7). This subnormal phenotype might come from genetic background, as UAS-CrzRRNAiS3s/+ heterozygous flies showed similar behavioral pattern to CrzR-KD flies. However, $CrzR^{mino}s$ flies did not test about their genetic background (Fig 6-7 E-F).

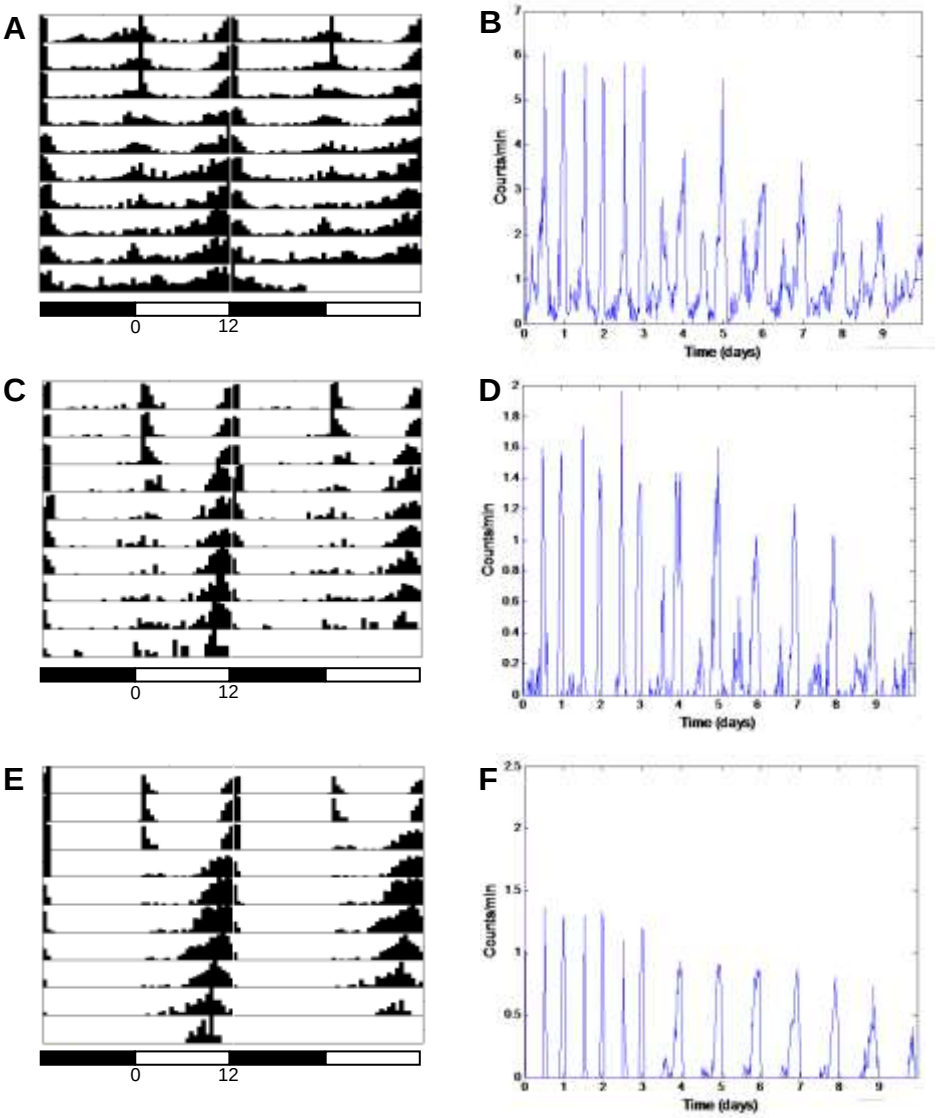


Fig. 6-5. Average actograms (left panel) and activity profiles (right panel). (A, B) *Crz-gal4*^{T2a} heterozygous flies. (C, D) *UAS-hid* heterozygous flies. (E, F) Crz-CD (*UAS-hid*/+; *Crz-gal4*/+) flies.

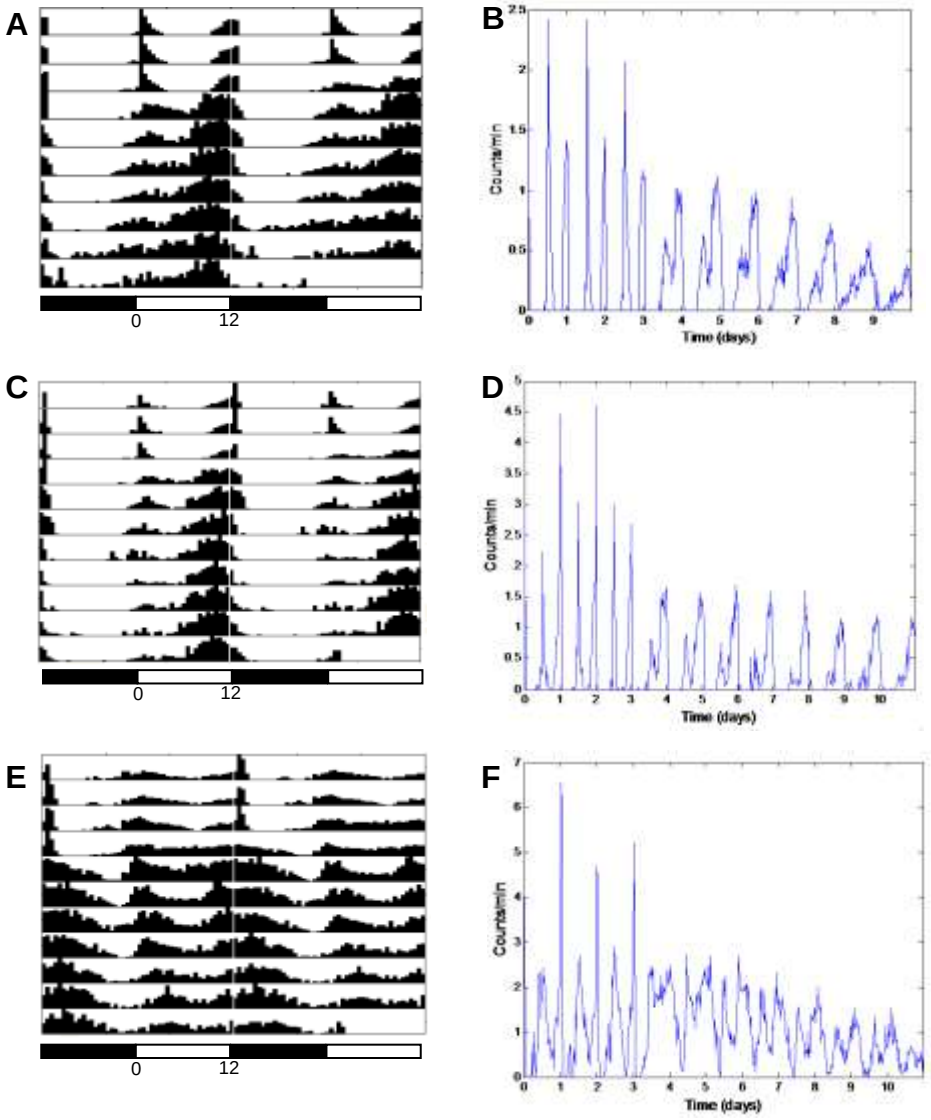


Fig. 6-6. Average actograms (left panel) and activity profiles (right panel). (A, B) Ectopic expression of *Crz* flies driven by *UAS-p35*. (C, D) Ectopic expression of *Crz* flies driven by *Pdf-gal4*. (E, F) Ectopic expression of *Crz* flies driven by *tim-gal4*.

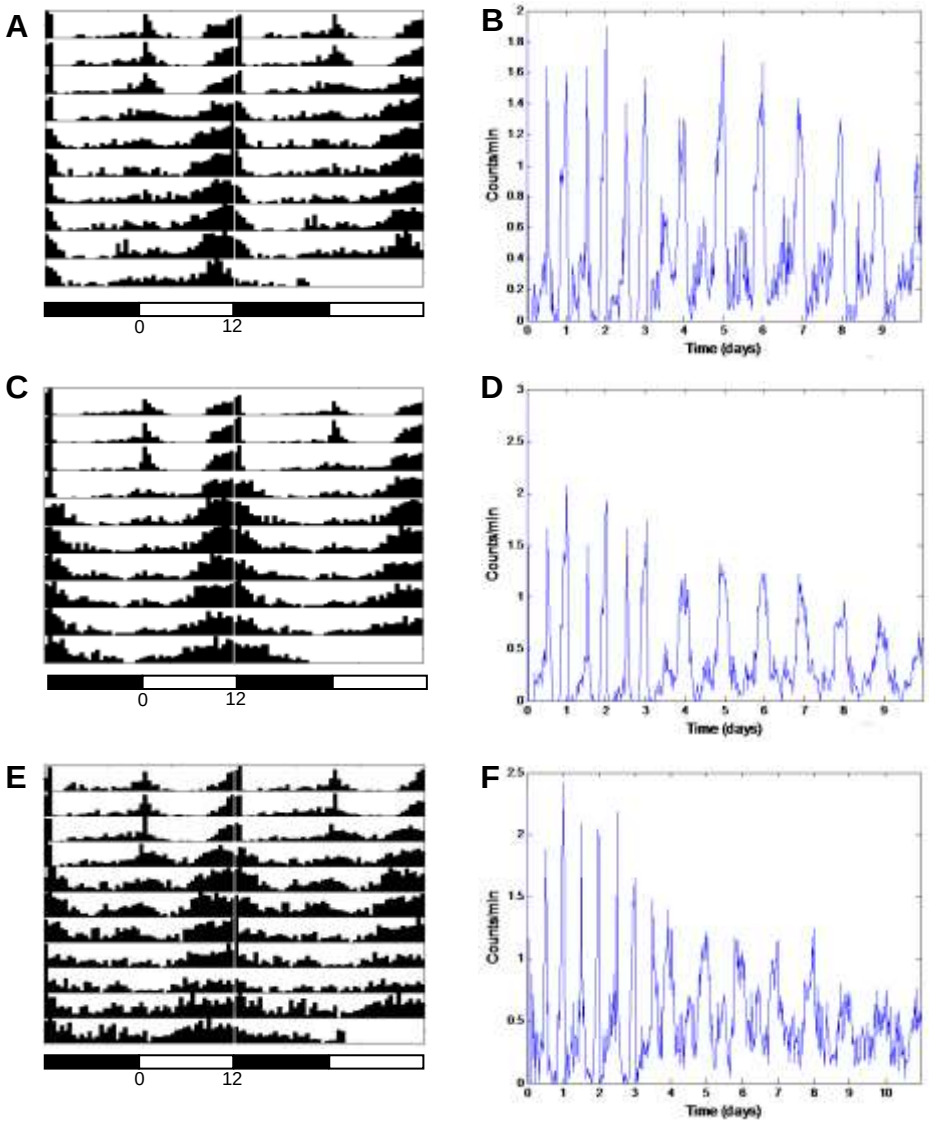


Fig. 6-7. Average actograms (left panel) and activity profiles (right panel). (A, B) *UAS-CrzR*^{RNAiS3S} heterozygous flies. (C, D) CrzR-KD flies. (E, F) *CrzR*^{minos} flies

The *pdf*^{01} flies showed severe abnormal behavior under DD conditions. *pdf*^{01} homozygote flies maintain their rhythmicity during 1-2 day in DD, but later on, flies became arrhythmic (Renn et al., 1999; Park, 2002). *Crz* soma locates nearby projection terminal of *Pdf* neurons (Choi et al., 2005). It might be possible that *Crz* is controlled by *Pdf* neurons and receive the biological clock information form *Pdf* neurons. If our hypothesis is correct, flies carrying *Pdf*^{01} mutation as well as Crz-CD showed more severe abnormal rhythmic locomotor activity than *Pdf*^{01} mutant flies. To test this possibility, we measured circadian rhythmic behavior of Crz-CD flies in *pdf*^{01} mutant background. To generate Crz-CD flies in *pdf*^{01} mutant background, we combine *Crz-gal4* or *UAS-rpr* with *pdf*^{01} mutant flies and then cross each other to eliminate *Crz* neurons. As expected, the parent flies expressed Crz, but did not express pdf (Fig. 6-8, A-F). However, the progeny did not express Crz and pdf both (Fig. 6-8, G-I). In contrast to our expectation, Crz-CD, *pdf*^{01} double mutant flies did not show synergistic effect (Fig. 6-9; summarized in Table 6-2). This result supports that *Crz* might not be important for the fly circadian rhythmic behavior.

Crz is not important for eclosion rhythmic behavior

Another well-characterized circadian rhythmic behavior is eclosion of pupae into adults. The circadian clocks regulate the timing of *Drosophila* eclosion, called gate, which occurs during early morning (Engelmann and Honegger, 1966). To test whether Crz involves eclosion rhythmicity, we measured eclosion of Crz-CD flies. Most of the adults emerged during morning time (Fig. 6-10). Crz-CD flies showed robust eclosion rhythms.

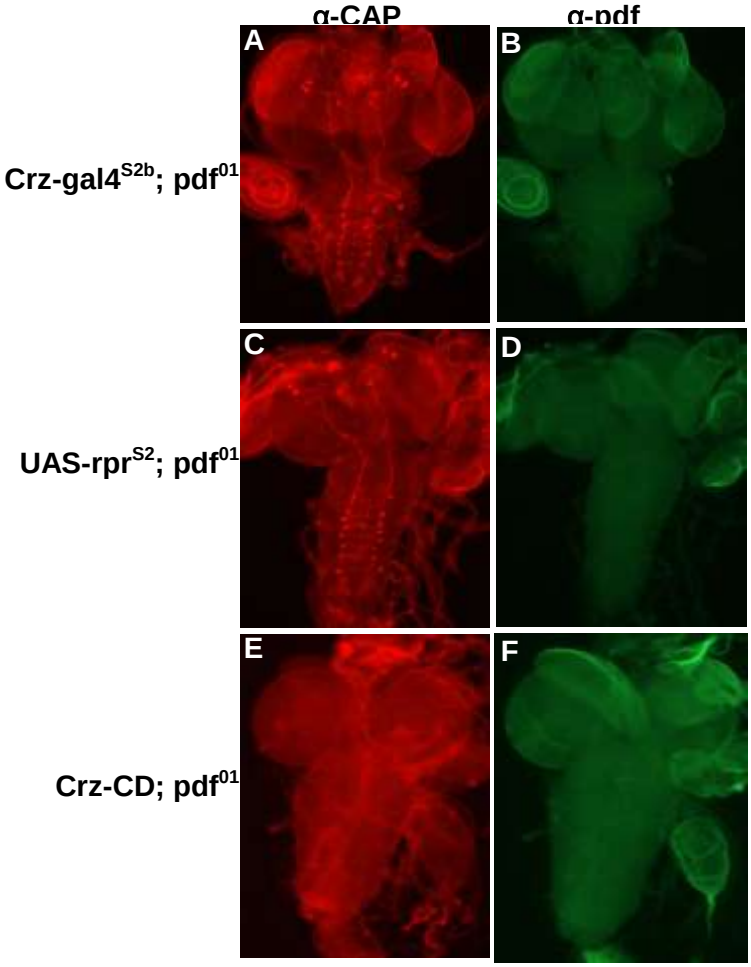


Fig. 6-8. Representative image of double immunohistochemistry in *pdf*^{01} mutant background. (A, C): Expression of *Crz* detected by anti-CAP in wandering larval stage, but not detected in the offspring from crossing *Crz-gal4*^{S2b}; *pdf*^{01} and *UAS-rpr*^{S2}; *pdf*^{01} (E). (B, D, and F): No expression of *pdf* confirmed by anti-pdf.

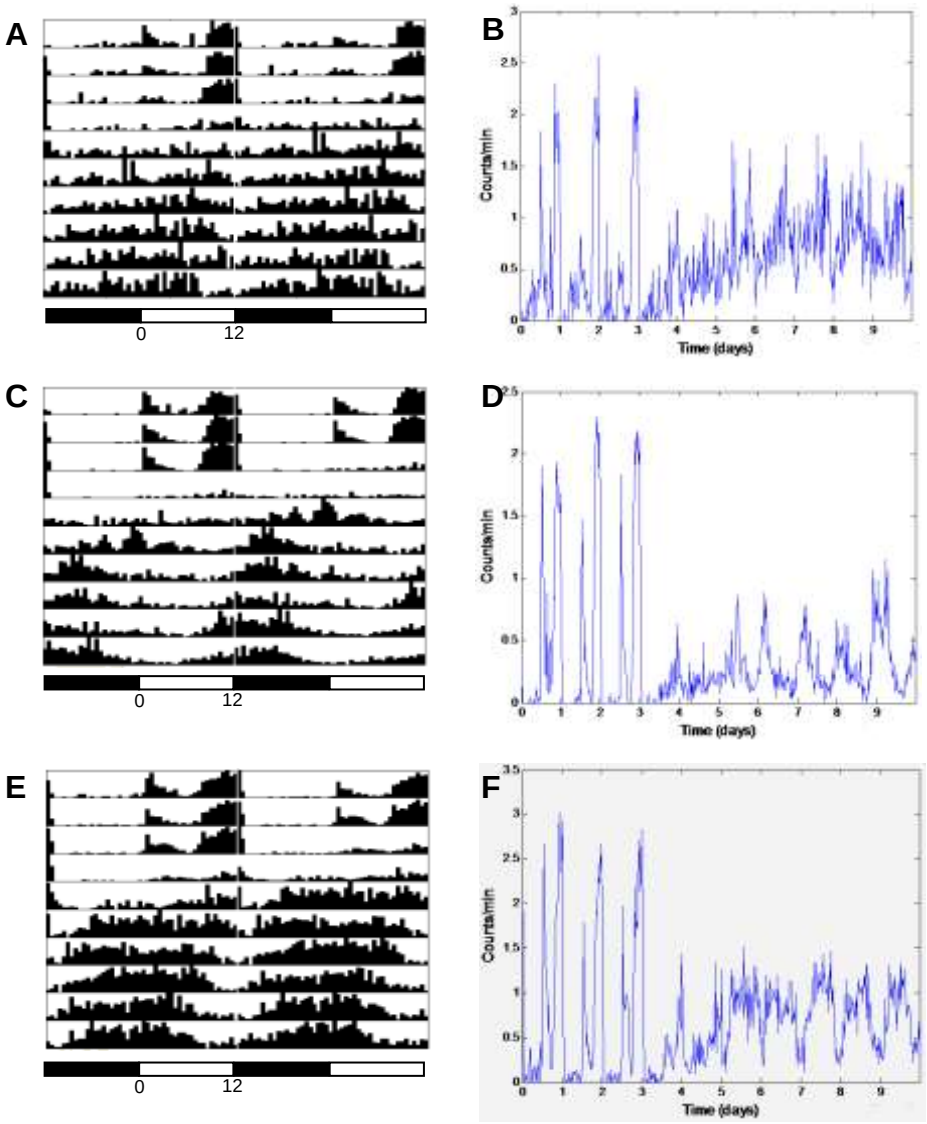


Fig. 6-9. Average actograms (left panel) and activity profiles (right panel) in *pdf*^{01} mutant backgruond. (A, B) *Crz-gal4*^{S2b}; *pdf*^{01} flies. (C, D) *UAS-rpr*^{S2}; *pdf*^{01} flies. (E, F) Crz-CD flies in *pdf*^{01} background

Table 6-2. Summary of locomotor activity rhythm in DD conditions

Genotype	n	R (%)	AR (%)	Period (hr) (mean±SEM)	Power (mean±SEM)
Crz-gal4^{T2a}/+	12	12 (100)	0 (0)	23.8±0.3	70.0±49.7
UAS-hid/+	13	12 (100)	0 (0)	23.7±0.3	66.1±42.7
Crz-CD	23	22 (95.7)	1 (4.3)	23.6±0.2	105.2±38.4
actin-gal4/+	14	14 (100)	0 (0)	24.5±0/5	47.7±26.7
UAS-CrzR^{RNAiS3s}/+	15	15 (100)	0 (0)	23.9±0.3	77.7±41.1
CrzR-KD	26	21 (80.8)	5 (19.2)	24.0±0.5	54.2±29.7
CrzR^{minos}	16	16 (100)	0 (0)	23.9±0.3	96.2±33.8
UAS-p35/*Crz-gal4*^{T2a}	27	26 (96.3)	1 (4.7)	23.8±0.4	84.5±38.9
UAS-Crz$^{\alpha}$/*pdf-gal4*	12	11 (91.7)	1 (8.3)	24.0±0.8	59.4±49.1
UAS-Crz$^{\alpha}$/*tim-gal4*	14	11 (79.6)	3 (20.4)	24.8±0.2	54.8±21.9
UAS-rpr^{S2};*pdf*^{01}	28	7 (25)	21 (75)	22.8±2.2	16.6±3.1
Crz-gal4^{S2b};*pdf*^{01}	31	8 (25.8)	23 (74.2)	23.9±3.4	39.7±27.5
Crz-CD;*pdf*^{01}	28	12 (42.9)	16 (57.1)	21.9±0.2	22.5±10.9

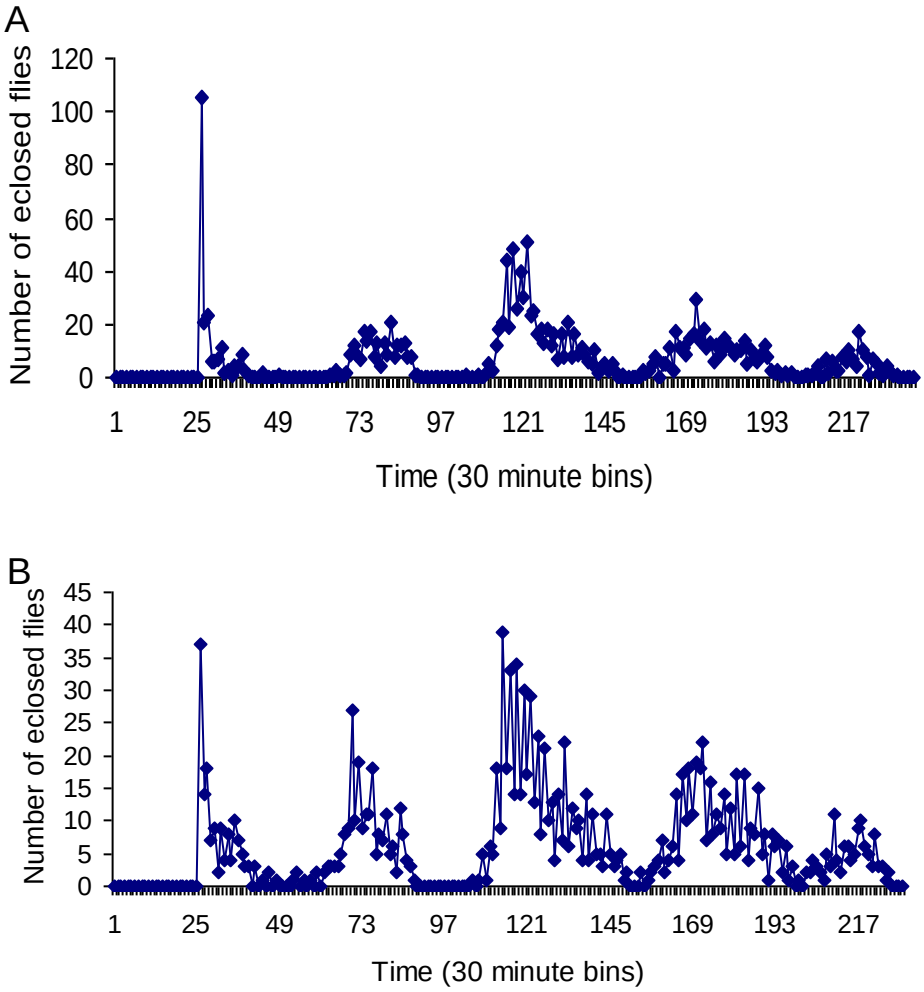


Fig. 6-10. Fly eclosion rhythmic behavior. (A) *Crz-gal4*^{T2a} heterozygous flies. (B) Crz-CD flies.

Discussion

In this study, we tried to characterize relationship between circadian rhythms and neuropeptide *Crz* in *Drosophila melanogaster*. The DLP-*Crz* neurons, in adult brain, locate in the vicinity of axon terminal of s-LNv-Pdf clock neurons (Choi et al., 2005). This result revises the possibility that *Crz* neurons are activated by pdf, when pdf peptides are released from the s-LNv axon terminals. If it is true, the activation of *Crz* neurons by pdf releasing might control circadian rhythmic behavior. However, we could not detect any defect in rhythmic behavior including free running locomotor behavior and eclosion behavior in Crz-CD, Ox-Crz, CrzR-KD, $CrzR^{minos}$ flies. These results suggest that to Crz is not important to detect the day-night timing transitions.

In female blow fly, *P. terraenovae* induces a reproductive diapause under short day low temperature condition, when the surgical removal of Crz-immunoreactive cells did not produce ovarian diapause under same conditions (Shiga and Numata, 2000). In another insect, *M. sexta*, pupal diapause can be induced by exposure of a short-day conditions, but surgical ablation of Crz-like immunoreactive neurons suppressed pupal diapause under LD=10h: 14h at 22℃ (Shiga et al., 2003). These results proposed that *Crz* is important to recognize seasonal transition instead of daily changes. To understand the relationship between seasonal changes in *Drosophila* and *Crz*, we also tested ovarian development in low temperature short day conditions. Nevertheless, we could not find any defect in ovarian development (data not shown).

Chapter Seven

General Conclusions and Future Directions

The main purpose of this research was to determine the regulation mechanisms and the functions of the neuropeptide *Crz in vivo*. Many studies have been performed in several insects to investigate the function of *Crz*; however, little is known about its function in *D. melanogaster*. In this study, the regulation mechanism of *Crz* was dissected by serial deletion of the promoter region using *UAS-GAL4* binary system. The functional analysis of *Crz* was approached using two different methods. One is elimination of *Crz* expression neurons by expression of proapoptotic protein in *Crz* neurons. The other is down-regulation of *CrzR* at the post-transcriptional level by RNA interference, which was generated by ubiquitous expression of *CrzR* double stranded RNA under control of *act-gal4* driver. Those studies validated the role of neuropeptide *Crz* in ethanol-related response, and olfactory behavior.

The transcriptional regulation of neuropeptide *Crz*

Previously, the expression pattern of *Crz* in *D. melanogaster* was determined by *in situ* hybridization and immunohistochemistry in larval and adult stages. In larval CNS, *Crz* expression is limited to 26 neurons out of ~ 10,000 CNS cells in three different

groups. In adult stage, *Crz* expression is dramatically changed; vCrz cells disappear by programmed cell death, DL cell numbers are increased from 3 to 6-8 pairs in each hemibrain (Choi et al., 2005). Four adult neurons in the abdominal ganglion express Crz in a gender-biased manner (Lee et al., 2008). The *Crz* regulatory site exists within a 1.2-kb upstream region (Choi et al., 2006).

In this study, we dissected *in vivo Crz* promoter activities to gain decisive insight into the tight regulatory mechanisms of *Crz* gene transcription. To elucidate the regulation mechanisms of *Crz* expression *in vivo* and in separate cell types, developmental stages, and gender-specific enhancers, we isolated the *Crz* 5' upstream regulatory region, fused it to the *gal4* coding sequence, and then, generated transgenic animals using *P* element mediated germ-line transformation. To detect promoter driven reporter gene expression, we used a yeast *UAS-gal4* binary system. Results of specific reporter gene expression suggested that *Crz* expression is driven by separate *cis*-acting elements. In addition, our data indicate that transcriptional regulatory mechanisms for *Crz* expression are not uniform among *Crz*-containing neurons, which further indicates that their neuronal functions might be different. As a result, we separated tissue, developmental stage, and gender-specific *cis*-acting elements. We shortened the *Crz*-regulatory region, which existed within 504-bp upstream sequence instead of 1.2-kb upstream. For larval DM expression, the region was located between -504-bp and -419-bp upstream. The other region between -380-bp and -241-bp is responsible for DL and vCrz expression in larval stages. For more insight into this region, DL has three elements and any two out of three combinations are needed for normal DL expression. In contrast, vCrz subdivides two elements and they are required altogether.

Interestingly, the basal transcription factor binding site, TATA box, has a minor role in *Crz* expression. For activating all adult expression, *Crz* needs only the -321-bp upstream region. The *cis*-element for male-specific abdominal ganglion is located in a region between -250-bp and -290-bp (summarized in Table 2-2 and 2-3).

Trans*-acting element for *Crz

Although specific *cis*-acting elements for *Crz* neurons have been elucidated, the responsible *trans*-acting factors are not well studied. Recently, transcription factors *nau* and *Mef2* were indentified as putative *Crz* promoter binding factors (Wentworth et al., 1991; Andres et al., 1995; Shiomi et al., 2005; Wei et al., 2007). To verify this possibility, we performed immunohistochemistry in trans-heterozygous *nau* null mutants and *Mef2* hypomorphic larva. Those two factors showed a minor effect in *Crz* neurons (Fig 2-18). Expression of another bHLH transcription factor, DIMM, colocalized in *Crz* neurons (Park et al., 2008). When DIMM is over-expressed by driving *Crz-gal4* driver in *Crz* neurons, *Crz* expression patterns are altered in a dose dependent manner. For example, one copy of DIMM enhances Crz immunoreactivity and increases DL cell number in larval stages, and protects from programmed cell death of vCrz during metamorphosis (Fig. 2-19). More than two copies of DIMM lead to translation of *Crz* in the optic medulla and change the cell fate in several brain neurons (Fig 2-20).

Crz* functions in the *D. melanogaster

Crz is multi-functional neuropeptide found in diverse insects. Several species specific functions are found; however, in the fruit fly, *D. melanogaster,* specific function

is still ambiguous. In locusts, synthetic Crz peptide injection in nymphal stage causes the reduction of the number of antennal sensilla in the adult stage (Maeno et al., 2004; Maeno and Tanaka, 2004). This result suggests that *Crz* is possibly involved in odor perception. In *Drosophila*, a *Crz* mutant is not available; therefore, we performed functional analysis in *Crz* neuronal deficient flies by proapoptotic protein expression in *Crz* neurons and *Crz receptor* knockdown flies by *CrzR* RNA interference expression in the whole animal. Interestingly, Crz-CD flies and CrzR-KD flies showed severe a defect in finding the source of complex volatile molecules generated from yeast compared to controls. With single molecules, Crz-CD and CrzR-KD flies showed lower attraction index. However, Crz-CD and CrzR-KD flies do not show any significant change of trichoid sensilla number. Those results strongly supported that Crz participates in modulation the olfactory perception mechanism, not in sensilla development.

Another possible function of *Crz* in *Drosophila* is ethanol related behavior. Crz-CD and CrzR-KD flies showed significantly delayed recovery from ethanol-induced sedation compared to control flies. This delayed recovery phenotype is ethanol-specific. Crz-CD and CrzR-KD flies do not show any effect on ethanol absorption and degradation. This result suggests that *Crz* is not involved in ADH regulation. Further investigation implies that Crz-CD and CrzR-KD flies do not affect the ALDH expression level; but rather reduce ALDH activity.

Crz neurons are located in the vicinity of nerve terminals from Pdf-expressing neurons. This observation suggests that Crz neurons might be part of the circadian rhythmic circuit. To demonstrate this possibility, we performed circadian rhythmic behavior and eclosion test. As results, Crz-CD and CrzR-KD flies is not show any effect

on circadian rhythmic behavior, suggesting that Crz neurons are not involved in this type of biological rhythm in *Drosophila*.

Future studies

The best way to understand the *in vivo* function of a gene is to study the mutant. Unfortunately, no neuropeptide *Crz* mutant is available. We attempted to generate *Crz* mutants by deletion of *Crz* gene locus using *P*-transposable element mobilization and by down-regulation of transcription levels using RNAi interference, but *Crz* mutant have not been obtained yet. Recently, we attempted another round of *P*-element mobilization and obtained a putative *Crz* null mutant. However, this putative *Crz* null mutant has another mutation in *pr-set7*, which is important for development. We are trying to rescue the *pr-set7* to obtain adult *Crz* mutant flies. Once *pr-set7* rescued flies are obtained, it will be examined whether *Crz* null mutant is involved in ethanol-related behavior and olfactory responses.

Previously, we purified several putative *trans*-acting factors to recognize *Crz cis*-acting sequence. However, those factors are not yet identified. After identification, those factors will be used in an *in vitro* binding assay with the *Crz* upstream sequence to find the relationship between regulation of *Crz* expression and factors by analysis of mutant phenotypes. We also found that the bHLH transcription factor DIMM controls *Crz* expression. Further investigation will be necessary to find the molecular mechanisms of DIMM in *Crz* neuronal development.

Literatures Cited

Agarwal, D. P. (2001). Genetic polymorphisms of alcohol metabolizing enzymes. *Pathol. Biol. (Paris)* **49**: 703-709.

Alcorta, E. (1991). Characterization of the electroantennogram in *Drosophila melanogaster* and its use for identifying olfactory capture and transduction mutants. *J. Neurophysiol.* **65**: 702-714.

Alcorta, E. and J. Rubio (1989). Intrapopulational variation of olfactory responses in *Drosophila melanogaster*. *Behav. Genet.* **19**: 285-299.

Allan, D. W., D. Park, S. E. St Pierre, P. H. Taghert and S. Thor (2005). Regulators acting in combinatorial codes also act independently in single differentiating neurons. *Neuron* **45**: 689-700.

Allan, D. W., S. E. St Pierre, I. Miguel-Aliaga and S. Thor (2003). Specification of neuropeptide cell identity by the integration of retrograde BMP signaling and a combinatorial transcription factor code. *Cell* **113**: 73-86.

Anderson, S. M. and S. E. Barnett (1991). The involvement of alcohol dehydrogenase and aldehyde dehydrogenase in alcohol/aldehyde metabolism in *Drosophila melanogaster*. *Genetica* **83**: 99-106.

Anderson, S. M., M. R. Brown and J. F. McDonald (1991). Tissue specific expression of the *Drosophila* Adh gene: a comparison of in situ hybridization and immunocytochemistry. *Genetica* **84**: 95-100.

Antonelli, A., L. Castellari, C. Zambonelli and A. Carnacini (1999). Yeast influence on volatile composition of wines. *J. Agric. Food Chem.* **47**: 1139-1144.

Arkhipova, I. R. (1995). Promoter elements in *Drosophila* melanogaster revealed by sequence analysis. *Genetics* **139**: 1359-1369.

Bae, K., X. Jin, E. S. Maywood, M. H. Hastings, S. M. Reppert and D. R. Weaver (2001). Differential functions of *mPer1*, *mPer2*, and *mPer3* in the SCN circadian clock. *Neuron* **30**: 525-536.

Baker, P. W., K. K. Tanaka, N. Klitgord and R. M. Cripps (2005). Adult myogenesis in *Drosophila melanogaste*r can proceed independently of myocyte enhancer factor-2. *Genetics* **170**: 1747-1759.

Balagopalan, L., C. A. Keller and S. M. Abmayr (2001). Loss-of-function mutations reveal that the *Drosophila nautilus* gene is not essential for embryonic myogenesis or viability. *Dev. Biol.* **231**: 374-382.

Bellen, H. J. (1998). The fruit fly: a model organism to study the genetics of alcohol abuse and addiction? *Cell* **93**: 909-912.

Bellen, H. J., R. W. Levis, G. Liao, Y. He, J. W. Carlson, G. Tsang, M. Evans-Holm, P. R. Hiesinger, K. L. Schulze, G. M. Rubin, R. A. Hoskins and A. C. Spradling (2004). The BDGP gene disruption project: single transposon insertions associated with 40% of *Drosophila* genes. *Genetics* **167**: 761-781.

Belmont, M., G. Cazzamali, M. Williamson, F. Hauser and C. J. Grimmelikhuijzen (2006). Identification of four evolutionarily related G protein-coupled receptors from the malaria mosquito *Anopheles gambiae*. *Biochem. Biophys. Res. Commun.* **344**: 160-165.

Benton, R. (2006). On the ORigin of smell: odorant receptors in insects. *Cell Mol. Life Sci.* **63**: 1579-1585.

Benton, R., S. Sachse, S. W. Michnick and L. B. Vosshall (2006). Atypical membrane topology and heteromeric function of *Drosophila* odorant receptors in vivo. *PLoS Biol.* **4**: e20.

Benton, R., K. S. Vannice, C. Gomez-Diaz and L. B. Vosshall (2009). Variant ionotropic glutamate receptors as chemosensory receptors in *Drosophila*. *Cell* **136**: 149-162.

Benveniste, R. J., S. Thor, J. B. Thomas and P. H. Taghert (1998). Cell type-specific regulation of the *Drosophila* FMRF-NH2 neuropeptide gene by Apterous, a LIM homeodomain transcription factor. *Development* **125**: 4757-4765.

Biggin, M. D. and R. Tjian (2001). Transcriptional regulation in *Drosophila*: the post-genome challenge. *Funct. Integr. Genomics* **1**: 223-234.

Bijlsma-Meeles, E. and R. Bijlsma (1988). The alcohol dehydrogenase polymorphism in *Drosophila melanogaster*: fitness measurements and predictions under conditions with no alcohol stress. *Genetics* **120**: 743-753.

Blau, J. and M. W. Young (1999). Cycling vrille expression is required for a functional *Drosophila* clock. *Cell* **99**: 661-671.

Blomqvist, A. G., C. Soderberg, I. Lundell, R. J. Milner and D. Larhammar (1992). Strong evolutionary conservation of neuropeptide Y: sequences of chicken, goldfish, and Torpedo marmorata DNA clones. *Proc. Natl. Acad. Sci. U S A* **89**: 2350-2354.

Bozza, T., P. Feinstein, C. Zheng and P. Mombaerts (2002). Odorant receptor expression defines functional units in the mouse olfactory system. *J. Neurosci.* **22**: 3033-3043.

Brand, A. H. and N. Perrimon (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118**: 401-415.

Brooks, W. M., L. N. Moxon, J. Field, M. G. Irving and D. M. Doddrell (1985). *In vitro* metabolism of [2-13C]-ethanol by 1H NMR spectroscopy using 13C decoupling with the reverse dept polarization-transfer pulse sequence. *Biochem. Biophys. Res. Commun.* **128**: 107-112.

Bross, T. G., B. Rogina and S. L. Helfand (2005). Behavioral, physical, and demographic changes in *Drosophila* populations through dietary restriction. *Aging Cell* **4**: 309-317.

Brown, M. R., J. W. Crim, R. C. Arata, H. N. Cai, C. Chun and P. Shen (1999). Identification of a *Drosophila* brain-gut peptide related to the neuropeptide Y family. *Peptides* **20**: 1035-1042.

Bruce, V. G. (1972). Mutants of the biological clock in *Chlamydomonas reinhardi*. *Genetics* **70**: 537-548.

Buck, L. and R. Axel (1991). A novel multigene family may encode odorant receptors: a molecular basis for odor recognition. *Cell* **65**: 175-87.

Bunning, E. (1954). Parental age and germinative characters of the seeds. *Ann. N Y Acad. Sci.* **57**: 484-487.

Bunning, E. and I. Moser (1968). Influence of water on the circadian rhythm of Phaseolus. *Naturwissenschaften* **55**: 450-451.

Casso, D., F. Ramirez-Weber and T. B. Kornberg (2000). GFP-tagged balancer chromosomes for *Drosophila melanogaster*. *Mech. Dev.* **91**: 451-454.

Cazzamali, G., N. Saxild and C. Grimmelikhuijzen (2002). Molecular cloning and functional expression of a *Drosophila* corazonin receptor. *Biochem. Biophys. Res. Commun.* **298**: 31-36.

Cermakian, N., L. Monaco, M. P. Pando, A. Dierich and P. Sassone-Corsi (2001). Altered behavioral rhythms and clock gene expression in mice with a targeted mutation in the *Period1* gene. *EMBO J.* **20**: 3967-3974.

Choi, Y. J., G. Lee, J. C. Hall and J. H. Park (2005). Comparative analysis of Corazonin-encoding genes (Crz's) in *Drosophila* species and functional insights into Crz-expressing neurons. *J. Comp. Neurol.* **482**: 372-385.

Choi, Y. J., G. Lee and J. H. Park (2006). Programmed cell death mechanisms of identifiable peptidergic neurons in *Drosophila melanogaster*. *Development* **133**: 2223-2232.

Clyne, P., A. Grant, R. O'Connell and J. R. Carlson (1997). Odorant response of individual sensilla on the *Drosophila* antenna. *Invert. Neurosci.* **3**: 127-135.

Clyne, P. J., S. J. Certel, M. de Bruyne, L. Zaslavsky, W. A. Johnson and J. R. Carlson (1999a). The odor specificities of a subset of olfactory receptor neurons are governed by Acj6, a POU-domain transcription factor. *Neuron* **22**: 339-347.

Clyne, P. J., C. G. Warr, M. R. Freeman, D. Lessing, J. Kim and J. R. Carlson (1999b). A novel family of divergent seven-transmembrane proteins: candidate odorant receptors in *Drosophila*. *Neuron* **22**: 327-338.

Crews, S. T. and C. M. Fan (1999). Remembrance of things PAS: regulation of development by bHLH-PAS proteins. *Curr Opin Genet Dev* **9**: 580-587.

Dahanukar, A., E. A. Hallem and J. R. Carlson (2005). Insect chemoreception. *Curr. Opin. Neurobiol.* **15**: 423-430.

Danner, S. and J. G. Belasco (2001). T7 phage display: a novel genetic selection system for cloning RNA-binding proteins from cDNA libraries. *Proc. Natl. Acad. Sci. U S A* **98**: 12954-12959.

David, J. R. and C. Bocquet (1975). Similarities and differences in latitudinal adaptation of two *Drosophila* sibling species. *Nature* **257**): 588-590.

David, J. R., C. Bocquet, M. F. Arens and P. Fouillet (1976). Biological role of alcohol dehydrogenase in the tolerance of *Drosophila melanogaster* to aliphatic alochols: utilization of an ADH-null mutant. *Biochem. Genet.* **14**: 989-997.

David, J. and J. Van Herrewege (1971). Fertility and laying behavior in *Drosophila melanogaster*: effect of several qualities of commercial yeast, of the volume of the cages and of the surface of the food. *Bull. Biol. Fr. Belg.* **105**: 345-356.

Davis, R. L. (2004). Olfactory learning. *Neuron* **44**: 31-48.

Davis, S. and S. Laroche (1996). Activation of metabotropic glutamate receptors induce differential effects on synaptic transmission in the dentate gyrus and CA1 of the hippocampus in the anaesthetized rat. *Neuropharmacology* **35**: 337-346.

Deutsch, J. C. and R. J. Nefdt (1992). Olfactory cues influence female choice in two lek-breeding antelopes. *Nature* **356**: 596-598.

Diamond, I. and A. S. Gordon (1997). Cellular and molecular neuroscience of alcoholism. *Physiol. Rev.* **77**: 1-20.

Dohrman, D. P., I. Diamond and A. S. Gordon (1997). The role of the neuromodulator adenosine in alcohol's actions. *Alcohol Health Res. World* **21**: 136-143.

Eisses, K. T., W. G. Schoonen, W. Aben, W. Scharloo and G. E. Thorig (1985). Dual function of the alcohol dehydrogenase of *Drosophila melanogaster*: ethanol and acetaldehyde oxidation by two allozymes ADH-71k and ADH-F. *Mol. Gen. Genet.* **199**: 76-81.

Eisses, K. T., W. G. Schoonen, W. Scharloo and G. E. Thorig (1985). Evidence for a multiple function of the alcohol dehydrogenase allozyme ADH71k of *Drosophila melanogaster*. *Comp. Biochem. Physiol. B* **82**: 863-868.

Elsaesser, R., G. Montani, R. Tirindelli and J. Paysan (2005). Phosphatidyl-inositide signalling proteins in a novel class of sensory cells in the mammalian olfactory epithelium. *Eur. J. Neurosci.* **21**: 2692-2700.

Engelmann, W. and H. W. Honegger (1966). Diurnal periodic sliding rhythm of an eyeless *Drosophila melanogaster* mutant. *Naturwissenschaften* **53**: 588.

Feingold, E. A., L. A. Penny, A. W. Nienhuis and B. G. Forget (1999). An olfactory receptor gene is located in the extended human beta-globin gene cluster and is expressed in erythroid cells. *Genomics* **61**: 15-23.

Feldman, J. F. and V. G. Bruce (1972). Circadian rhythm changes in autotrophic euglena induced by organic carbon sources. *J. Protozool.* **19**: 370-373.

Feldman, J. F. and M. N. Hoyle (1973). Isolation of circadian clock mutants of *Neurospora crassa*. *Genetics* **75**: 605-613.

Fibla, J., L. Enjuanes and R. Gonzalez-Duarte (1989). Inter-specific analysis of *Drosophila* alcohol dehydrogenase by an immunoenzymatic assay using monoclonal antibodies. *Biochem. Biophys. Res. Commun.* **160**: 638-646.

Firestein, S. (2001). How the olfactory system makes sense of scents. *Nature* **413**: 211-218.

Freitag, J., J. Krieger, J. Strotmann and H. Breer (1995). Two classes of olfactory receptors in *Xenopus laevis*. *Neuron* **15**: 1383-1392.

Freriksen, A., D. Seykens, W. Scharloo and P. W. Heinstra (1991). Alcohol dehydrogenase controls the flux from ethanol into lipids in *Drosophila* larvae. A 13C NMR study. *J. Biol. Chem.* **266**: 21399-21403.

Fry, J. D., C. M. Bahnck, M. Mikucki, N. Phadnis and W. C. Slattery (2004). Dietary Ethanol Mediates Selection on Aldehyde Dehydrogenase Activity in *Drosophila melanogaster*. *Interg. Comp. Biol.* **44**: 275-283.

Fry, J. D. and M. Saweikis (2006). Aldehyde dehydrogenase is essential for both adult and larval ethanol resistance in Drosophila melanogaster. *Genet. Res.* **87**: 87-92.

Garcin, F., J. Cote, G. Lau You Hin, S. Chawla and S. Radouco-Thomas (1986). Ethanol metabolizing system in *Drosophila*. Aldehyde dehydrogenase: functional aspects in adult and during development. *Prog. Neuropsychopharmacol. Biol. Psychiatry* **10**: 167-176.

Garczynski, S. F., M. R. Brown, P. Shen, T. F. Murray and J. W. Crim (2002). Characterization of a functional neuropeptide F receptor from *Drosophila melanogaster*. **Peptides 23:** 773-780.

Gauthier, S. A. and R. S. Hewes (2006). Transcriptional regulation of neuropeptide and peptide hormone expression by the *Drosophila* dimmed and cryptocephal genes. *J. Exp. Biol.* **209**): 1803-1815.

Geer, B. W., M. L. Langevin and S. W. McKechnie (1985). Dietary ethanol and lipid synthesis in *Drosophila melanogaster*. *Biochem. Genet.* **23**: 607-622.

Gendre, N., K. Luer, S. Friche, N. Grillenzoni, A. Ramaekers, G. M. Technau and R. F. Stocker (2004). Integration of complex larval chemosensory organs into the adult nervous system of *Drosophila*. *Development* **131**: 83-92.

Gibson, J. B. and J. G. Oakeshott (1981). Genetics of biochemical and behavioural aspects of alcohol metabolism. *Aust. N Z J. Med.* **11**: 128-131.

Giordano, E., R. Rendina, I. Peluso and M. Furia (2002). RNAi triggered by symmetrically transcribed transgenes in *Drosophila melanogaster. Genetics* **160**: 637-648.

Glossop, N. R., L. C. Lyons and P. E. Hardin (1999). Interlocked feedback loops within the *Drosophila* circadian oscillator. *Science* **286**: 766-768.

Glusman, G., A. Bahar, D. Sharon, Y. Pilpel, J. White and D. Lancet (2000). The olfactory receptor gene superfamily: data mining, classification, and nomenclature. *Mamm. Genome*. **11**: 1016-1023.

Glusman, G., I. Yanai, I. Rubin and D. Lancet (2001). The complete human olfactory subgenome. *Genome Res.* **11**: 685-702.

Gonzales, R. A., M. O. Job and W. M. Doyon (2004). The role of mesolimbic dopamine in the development and maintenance of ethanol reinforcement. *Pharmacol. Ther.* **103**: 121-146.

Grover, C. A., K. A. Wallace, S. A. Lindberg and G. D. Frye (1998). Ethanol inhibition of NMDA currents in acutely dissociated medial septum/diagonal band neurons from ethanol dependent rats. *Brain Res*. **782**: 43-52.

Grimmelikhuijzen, C. J., I. Leviev and K. Carstensen (1996). Peptides in the nervous systems of cnidarians: structure, function, and biosynthesis. *Int. Rev. Cytol.* **167**: 37-89.

Guarnieri, D. J. and U. Heberlein (2003). *Drosophila melanogaster*, a genetic model system for alcohol research. *Int. Rev. Neurobiol.* **54**: 199-228.

Hallem, E. A. and J. R. Carlson (2004a). The odor coding system of *Drosophila*. *Trends Genet*. **20**: 453-459.

Hallem, E. A. and J. R. Carlson (2004b). The spatial code for odors is changed by conditioning. *Neuron* **42**: 359-361.

Hallem, E. A. and J. R. Carlson (2006). Coding of odors by a receptor repertoire. *Cell* **125**: 143-160.

Hallem, E. A., A. Dahanukar and J. R. Carlson (2006). Insect odor and taste receptors. *Annu. Rev. Entomol.* **51**: 113-135.

Hallem, E. A., M. G. Ho and J. R. Carlson (2004). The molecular basis of odor coding in the *Drosophila* antenna. *Cell* **117**): 965-979.

Halligan, D. L. and P. D. Keightley (2006). Ubiquitous selective constraints in the *Drosophila* genome revealed by a genome-wide interspecies comparison. *Genome Res.* **16**: 875-884.

Hamblen, M., W. A. Zehring, C. P. Kyriacou, P. Reddy, Q. Yu, D. A. Wheeler, L. J. Zwiebel, R. J. Konopka, M. Rosbash and J. C. Hall (1986). Germ-line transformation involving DNA from the period locus in *Drosophila melanogaster*: overlapping genomic fragments that restore circadian and ultradian rhythmicity to per0 and per- mutants. *J. Neurogenet.* **3**: 249-291.

Hansen, I. A., F. Sehnal, S. R. Meyer and K. Scheller (2001). Corazonin gene expression in the waxmoth *Galleria mellonella*. *Insect Mol. Biol*. **10**: 341-346.

Hao, H., N. R. Glossop, L. Lyons, J. Qiu, B. Morrish, Y. Cheng, C. Helfrich-Forster and P. Hardin (1999). The 69 bp circadian regulatory sequence (CRS) mediates per-like developmental, spatial, and circadian expression and behavioral rescue in *Drosophila*. *J. Neurosci.* **19**: 987-994.

Hartenstein (1993). Atlas of *Drosophila* development. *Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY*

Hauser, F., G. Cazzamali, M. Williamson, W. Blenau and C. J. Grimmelikhuijzen (2006). A review of neurohormone GPCRs present in the fruitfly *Drosophila melanogaster* and the honey bee *Apis mellifera*. *Prog. Neurobiol.* **80**: 1-19.

Heberlein, U. (2000). Genetics of alcohol-induced behaviors in *Drosophila*. *Alcohol Res*. *Health* **24:** 185-188.

Heinstra, P. W. (1993). Evolutionary genetics of the *Drosophila* alcohol dehydrogenase gene-enzyme system. *Genetica* **92**: 1-22.

Heinstra, P. W., W. J. Aben, W. Scharloo and G. E. Thorig (1986). Alcohol dehydrogenase of *Drosophila melanogaster*: metabolic differences mediated through cryptic allozymes. *Heredity* **57**: 23-29.

Heinstra, P. W. and B. W. Geer (1991). Metabolic control analysis and enzyme variation: nutritional manipulation of the flux from ethanol to lipids in *Drosophila*. *Mol. Biol. Evol.* **8**: 703-708.

Heinstra, P. W., B. W. Geer, D. Seykens and M. Langevin (1989). The metabolism of ethanol-derived acetaldehyde by alcohol dehydrogenase (EC 1.1.1.1) and

aldehyde dehydrogenase (EC 1.2.1.3) in *Drosophila melanogaster* larvae. *Biochem. J.* **259**: 791-797.

Heinstra, P. W., W. Scharloo and G. E. Thorig (1987). Physiological significance of the alcohol dehydrogenase polymorphism in larvae of *Drosophila*. *Genetics* **117**: 75-84.

Heinz, A., M. Schafer, J. D. Higley, J. H. Krystal and D. Goldman (2003). Neurobiological correlates of the disposition and maintenance of alcoholism. *Pharmacopsychiatry* **36 Suppl 3**: S255-258.

Helfand, S. L. and J. R. Carlson (1989). Isolation and characterization of an olfactory mutant in *Drosophila* with a chemically specific defect. *Proc. Natl. Acad. Sci. U S A* **86**: 2908-2912.

Helfrich-Forster, C. (2005). PDF has found its receptor. *Neuron* **48**: 161-163.

Hewes, R. S. and P. H. Taghert (2001). Neuropeptides and neuropeptide receptors in the *Drosophila melanogaster* genome*. Genome Res.* **11**: 1126-1142.

Higuchi, H., A. Hasegawa and T. Yamaguchi (2005). Transcriptional regulation of neuronal genes and its effect on neural functions: transcriptional regulation of neuropeptide Y gene by leptin and its effect on feeding. *J. Pharmacol. Sci.* **98**: 225-231.

Hill, C. A., A. N. Fox, R. J. Pitts, L. B. Kent, P. L. Tan, M. A. Chrystal, A. Cravchik, F. H. Collins, H. M. Robertson and L. J. Zwiebel (2002a). G protein-coupled receptors in *Anopheles gambiae*. *Science* **298:** 176-178.

Hill, E. S., M. Iwano, L. Gatellier and R. Kanzaki (2002b). Morphology and physiology of the serotonin-immunoreactive putative antennal lobe feedback neuron in the male silkmoth *Bombyx mori*. *Chem. Senses* **27**: 475-483.

Hinson, J. A. and R. A. Neal (1972). An examination of the oxidation of aldehydes by horse liver alcohol dehydrogenase. *J. Biol. Chem.* **247:** 7106-7107.

Hollingsworth, M. J. and J. V. Burcombe (1970). The nutritional requirements for longevity in *Drosophila*. *J. Insect. Physiol.* **16**: 1017-1025.

Holzenberger, M., F. Lapointe, M. Leibovici and C. A. Lievre (1996). The avian IGF type 1 receptor: cDNA analysis and in situ hybridization reveal conserved sequence elements and expression patterns relevant for the development of the nervous system. *Brain Res. Dev. Brain Res.* **97**: 76-87.

Hua, Y., J. Ishibashi, H. Saito, A. I. Tawfik, M. Sakakibara, Y. Tanaka, R. Derua, E. Waelkens, G. Baggerman, A. De Loof, L. Schoofs and S. Tanaka (2000). Identification of [Arg^7] corazonin in the silkworm, *Bombyx mori* and the cricket, *Gryllus bimaculatus*, as a factor inducing dark color in an albino strain of the locust,*Locusta migratoria*. *J. Insect. Physiol.* **46**: 853-860.

Imai, T. and H. Sakano (2008). Odorant receptor-mediated signaling in the mouse. *Curr. Opin. Neurobiol.* **18**: 251-260.

Impraim, C., G. Wang and A. Yoshida (1982). Structural mutation in a major human aldehyde dehydrogenase gene results in loss of enzyme activity. *Am. J. Hum. Genet.* **34**: 837-841.

Isse, T., K. Matsuno, T. Oyama, K. Kitagawa and T. Kawamoto (2005). Aldehyde dehydrogenase 2 gene targeting mouse lacking enzyme activity shows high

acetaldehyde level in blood, brain, and liver after ethanol gavages. *Alcohol Clin. Exp. Res.* **29**: 1959-1964.

Johnson, E. C., O. T. Shafer, J. S. Trigg, J. Park, D. A. Schooley, J. A. Dow and P. H. Taghert (2005). A novel diuretic hormone receptor in *Drosophila*: evidence for conservation of CGRP signaling. *J. Exp. Biol.* **208**: 1239-1246.

Kadonaga, J. T. (2004). Regulation of RNA polymerase II transcription by sequence-specific DNA binding factors. *Cell* **116**: 247-257.

Kaneko, M., J. H. Park, Y. Cheng, P. E. Hardin and J. C. Hall (2000). Disruption of synaptic transmission or clock-gene-product oscillations in circadian pacemaker cells of *Drosophila* cause abnormal behavioral rhythms. *J. Neurobiol.* **43**: 207-233.

Karachentsev, D., K. Sarma, D. Reinberg and R. Steward (2005). *PR-Se*t7-dependent methylation of histone H4 Lys 20 functions in repression of gene expression and is essential for mitosis. *Genes Dev.* **19**: 431-435.

Ke, S. H. and E. L. Madison (1997). Rapid and efficient site-directed mutagenesis by single-tube 'megaprimer' PCR method. *Nucleic Acids Res.* **25**: 3371-3372.

Keene, A. C. and S. Waddell (2007). *Drosophila* olfactory memory: single genes to complex neural circuits. *Nat. Rev. Neurosci.* **8**: 341-354.

Keightley, P. D. and T. Johnson (2004). MCALIGN: stochastic alignment of noncoding DNA sequences based on an evolutionary model of sequence evolution. *Genome Res.* **14**: 442-450.

Kim, M. S. and D. P. Smith (2001). The invertebrate odorant-binding protein LUSH is required for normal olfactory behavior in *Drosophila*. *Chem. Senses* **26**: 195-199.

Kim, Y. J., I. Spalovska-Valachova, K. H. Cho, I. Zitnanova, Y. Park, M. E. Adams and D. Zitnan (2004). Corazonin receptor signaling in ecdysis initiation. *Proc. Natl. Acad. Sci. U S A* **101**: 6704-6709.

Konopka, R. J. and S. Benzer (1971). Clock mutants of *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. U S A* **68**: 2112-2116.

Kutach, A. K. and J. T. Kadonaga (2000). The downstream promoter element DPE appears to be as widely used as the TATA box in *Drosophila* core promoters. *Mol. Cell. Biol.* **20**: 4754-4764.

Larsson, M. C., A. I. Domingos, W. D. Jones, M. E. Chiappe, H. Amrein and L. B. Vosshall (2004). *Or83b* encodes a broadly expressed odorant receptor essential for *Drosophila* olfaction. *Neuron* **43**: 703-714.

Leal, J. F. and M. Barbancho (1993). Aldehyde dehydrogenase (ALDH) activity in *Drosophila melanogaster* adults: evidence for cytosolic localization. *Insect Biochem. Mol. Biol.* **23**: 543-547.

Lee, C., J. P. Etchegaray, F. R. Cagampang, A. S. Loudon and S. M. Reppert (2001). Posttranslational mechanisms regulate the mammalian circadian clock. *Cell* **107**: 855-867.

Lee, G., J. H. Bahn and J. H. Park (2006). Sex- and clock-controlled expression of the neuropeptide F gene in *Drosophila*. *Proc. Natl. Acad. Sci. U S A* **103**: 12580-12585.

Lee, G., K. M. Kim, K. Kikuno, Z. Wang, Y. J. Choi and J. H. Park (2008). Developmental regulation and functions of the expression of the neuropeptide corazonin in *Drosophila melanogaster*. *Cell Tissue Res.* **331**: 659-673.

Lee, H. G., Y. C. Kim, J. S. Dunning and K. A. Han (2008). Recurring ethanol exposure induces disinhibited courtship in *Drosophila*. PLoS ONE **3**: e1391.

Lee, G. and J. H. Park (2004). Hemolymph sugar homeostasis and starvation-induced hyperactivity affected by genetic manipulations of the adipokinetic hormone-encoding gene in *Drosophila melanogaster*. *Genetics* **167**: 311-323.

Lee, K. A. (2001). Sleep and fatigue. *Annu. Rev. Nurs. Res.* **19**: 249-273.

Lee, T. and L. Luo (1999). Mosaic analysis with a repressible cell marker for studies of gene function in neuronal morphogenesis. *Neuron* **22**: 451-461.

Lei, H., J. A. Riffell, S. L. Gage and J. G. Hildebrand (2009). Contrast enhancement of stimulus intermittency in a primary olfactory network and its behavioral significance. *J. Biol* .**8**: 21.

Leibovici, M., F. Lapointe, P. Aletta and C. Ayer-Le Lievre (1996). Avian olfactory receptors: differentiation of olfactory neurons under normal and experimental conditions. *Dev. Biol.* **175**: 118-131.

Li, L., W. P. Kelley, C. P. Billimoria, A. E. Christie, S. R. Pulver, J. V. Sweedler and E. Marder (2003). Mass spectrometric investigation of the neuropeptide complement and release in the pericardial organs of the crab, Cancer borealis. *J. Neurochem.* **87**: 642-656.

Li, A. J., Ozawa, K., Tsuboyama, H., and Imamura, T. (1999) Distribution of fibroblast growth factor-5 in rat hypothalamus, and its possible role as a regulator of feeding behaviour. *Eur. J. Neurosci.* **11***:* 1362-1368

Libert, S., J. Zwiener, X. Chu, W. Vanvoorhies, G. Roman and S. D. Pletcher (2007). Regulation of *Drosophila* life span by olfaction and food-derived odors. *Science* **315**: 1133-1137.

Lietaert, M. C., M. Libion-Mannaert, S. Wattiaux-De Coninck and A. Elens (1985). *Drosophila melanogaster* aldehyde dehydrogenase. *Experientia* **41**: 57-58.

Lilly, B., B. Zhao, G. Ranganayakulu, B. M. Paterson, R. A. Schulz and E. N. Olson (1995). Requirement of MADS domain transcription factor D-MEF2 for muscle formation in *Drosophila*. *Science* **267**: 688-693.

Liu, F., G. Baggerman, W. D'Hertog, P. Verleyen, L. Schoofs and G. Wets (2006). *In silico* identification of new secretory peptide genes in *Drosophila melanogaster*. *Mol. Cell. Proteomics* **5**: 510-522.

Lovinger, D. M. and J. C. Crabbe (2005). Laboratory models of alcoholism: treatment target identification and insight into mechanisms. *Nat. Neurosci.* **8**: 1471-1480.

Luan, H., W. C. Lemon, N. C. Peabody, J. B. Pohl, P. K. Zelensky, D. Wang, M. N. Nitabach, T. C. Holmes and B. H. White (2006). Functional dissection of a neuronal network required for cuticle tanning and wing expansion in *Drosophila*. *J. Neurosci.* **26**: 573-584.

Maeno, K., T. Gotoh and S. Tanaka (2004). Phase-related morphological changes induced by [His^7]-corazonin in two species of locusts, *Schistocerca gregaria* and *Locusta migratoria* (Orthoptera: Acrididae). *Bull. Entomol. Res.* **94**: 349-357.

Maeno, K. and S. Tanaka (2004). Hormonal control of phase-related changes in the number of antennal sensilla in the desert locust, *Schistocerca gregaria*: possible involvement of [His^7]-corazonin. *J. Insect. Physiol.* **50**: 855-865.

Marrus, S. B., H. Zeng and M. Rosbash (1996). Effect of constant light and circadian entrainment of *perS* flies: evidence for light-mediated delay of the negative feedback loop in *Drosophila*. *EMBO J.* **15**: 6877-6886.

Martinek, S., S. Inonog, A. S. Manoukian and M. W. Young (2001). A role for the segment polarity gene shaggy/GSK-3 in the *Drosophila* circadian clock. *Cell* **105**: 769-779.

Martinek, S. and M. W. Young (2000). Specific genetic interference with behavioral rhythms in *Drosophila* by expression of inverted repeats. *Genetics* **156**: 1717-1725.

Mayer, M. L. and N. Armstrong (2004). Structure and function of glutamate receptor ion channels. *Annu. Rev. Physiol.* **66**: 161-181.

McKenna, M., P. Monte, S. L. Helfand, C. Woodard and J. Carlson (1989). A simple chemosensory response in *Drosophila* and the isolation of acj mutants in which it **is affected. *Proc. Natl. Acad. Sci. U S A* 86: 8118-8122.**

McKenzie, J. A. and S. W. McKechnie (1978). Ethanol tolerance and the Adh polymorphism in a natural population of *Drosophila melanogaster*. *Nature* **272**: 75-76.

Menco, B. P. and J. E. Jackson (1997). A banded topography in the developing rat's olfactory epithelial surface. *J. Comp. Neurol.* **388**: 293-306.

Mercot, H. (1994). Phenotypic expression of ADH regulatory genes in *Drosophila melanogaster*: a comparative study between a paleartic and a tropical population. *Genetica* **94**: 37-41.

Mizoi, Y., K. Yamamoto, Y. Ueno, T. Fukunaga and S. Harada (1994). Involvement of genetic polymorphism of alcohol and aldehyde dehydrogenases in individual variation of alcohol metabolism. *Alcohol Alcohol* **29**: 707-710.

Mombaerts, P. (1999). Molecular biology of odorant receptors in vertebrates. *Annu. Rev. Neurosci.* **22**: 487-509.

Moore, M. S., J. DeZazzo, A. Y. Luk, T. Tully, C. M. Singh and U. Heberlein (1998). Ethanol intoxication in *Drosophila*: Genetic and pharmacological evidence for regulation by the cAMP signaling pathway. *Cell* **93**: 997-1007.

Naidoo, N., W. Song, M. Hunter-Ensor and A. Sehgal (1999). A role for the proteasome in the light response of the timeless clock protein. *Science* **285**: 1737-1741.

Nassel, D. R. (2002). Neuropeptides in the nervous system of *Drosophila* and other insects: multiple roles as neuromodulators and neurohormones. *Prog. Neurobiol.* **68**: 1-84.

Nef, P., I. Hermans-Borgmeyer, H. Artieres-Pin, L. Beasley, V. E. Dionne and S. F. Heinemann (1992). Spatial pattern of receptor expression in the olfactory epithelium. *Proc. Natl. Acad. Sci. U S A* **89**: 8948-8952.

Nestler, E. J. (2005). Is there a common molecular pathway for addiction? *Nat. Neurosci.* **8**: 1445-1449.

Nestler, E. J. and G. K. Aghajanian (1997). Molecular and cellular basis of addiction. *Science* **278**: 58-63.

Neuhaus, E. M., G. Gisselmann, W. Zhang, R. Dooley, K. Stortkuhl and H. Hatt (2005). Odorant receptor heterodimerization in the olfactory system of *Drosophila melanogaster*. *Nat. Neurosci.* **8**: 15-17.

Ngai, J., A. Chess, M. M. Dowling, N. Necles, E. R. Macagno and R. Axel (1993a). Coding of olfactory information: topography of odorant receptor expression in the catfish olfactory epithelium. *Cell* **72**: 667-680.

Ngai, J., M. M. Dowling, L. Buck, R. Axel and A. Chess (1993b). The family of genes encoding odorant receptors in the channel catfish. *Cell* **72**: 657-666.

Noyes, B. E., F. N. Katz and M. H. Schaffer (1995). Identification and expression of the *Drosophila* adipokinetic hormone gene. *Mol. Cell. Endocrinol.* **109**: 133-141.

Oudman, L., W. Van Delden, A. Kamping and R. Bijlsma (1991). Polymorphism at the Adh and alpha Gpdh loci in *Drosophila melanogaster*: effects of rearing temperature on developmental rate, body weight, and some biochemical parameters. *Heredity* **67**: 103-115.

Ouyang, Y., C. R. Andersson, T. Kondo, S. S. Golden and C. H. Johnson (1998). Resonating circadian clocks enhance fitness in cyanobacteria. *Proc. Natl. Acad. Sci. U S A* **95**: 8660-8664.

Park, D., J. A. Veenstra, J. H. Park and P. H. Taghert (2008). Mapping peptidergic cells in *Drosophila*: where DIMM fits in. *PLoS ONE* **3**: e1896.

Park, J. H. (2002). Downloading central clock information in *Drosophila*. *Mol. Neurobiol.* **26**: 217-233.

Park, J. H. and J. C. Hall (1998). Isolation and chronobiological analysis of a neuropeptide pigment-dispersing factor gene in *Drosophila melanogaster*. *J. Biol. Rhythms* **13**: 219-228.

Park, J. H., C. Helfrich-Forster, G. Lee, L. Liu, M. Rosbash and J. C. Hall (2000). Differential regulation of circadian pacemaker output by separate clock genes in *Drosophila*. *Proc. Natl. Acad. Sci. U S A* **97**: 3608-3613.

Parsons, P. A. and S. B. King (1977). Ethanol: larval discrimination between two *Drosophila* sibling species. *Experientia* **33**: 898-899.

Paterson, B. M., U. Walldorf, J. Eldridge, A. Dubendorfer, M. Frasch and W. J. Gehring (1991). The *Drosophila* homologue of vertebrate myogenic-determination genes encodes a transiently expressed nuclear protein marking primary myogenic cells. *Proc. Natl. Acad. Sci. U S A* **88**: 3782-3786.

Pavan, C., T. Dobzhansky and A. B. da Cunha (1957). HETEROSIS AND ELIMINATION OF WEAK HOMOZYGOTES IN NATURAL POPULATIONS OF THREE RELATED SPECIES OF *Drosophila*. *Proc. Natl. Acad. Sci. U S A* **43**: 226-234.

Pecsenye, K. and A. Saura (1998). Interaction between the Adh and Odh loci in response to ethanol in *Drosophila melanogaster*. *Biochem. Genet***. 36**: 147-170.

Peng, G. S., M. F. Wang, C. Y. Chen, S. U. Luu, H. C. Chou, T. K. Li and S. J. Yin (1999). Involvement of acetaldehyde for full protection against alcoholism by homozygosity of the variant allele of mitochondrial aldehyde dehydrogenase gene in Asians. *Pharmacogenetics* **9**: 463-476.

Pifferi, S., G. Pascarella, A. Boccaccio, A. Mazzatenta, S. Gustincich, A. Menini and S. Zucchelli (2006). Bestrophin-2 is a candidate calcium-activated chloride channel involved in olfactory transduction. *Proc. Natl. Acad. Sci. U S A* **103**: 12929-12934.

Pitts, R. J., A. N. Fox and L. J. Zwiebel (2004). A highly conserved candidate chemoreceptor expressed in both olfactory and gustatory tissues in the malaria vector *Anopheles gambiae*. *Proc. Natl. Acad. Sci. U S A* **101**: 5058-5063.

Pitts, R. J. and L. J. Zwiebel (2006). Antennal sensilla of two female anopheline sibling species with differing host ranges. *Malar. J.* **5**: 26.

Pletcher, S. D. and M. P. Stumpf (2002). Population genomics: ageing by association. *Curr. Biol.* **12**: R328-330.

Powell, J. R. and E. N. Moriyama (1997). Evolution of codon usage bias in *Drosophila*. *Proc. Natl. Acad. Sci. U S A* **94**: 7784-7790.

Price, J. L., J. Blau, A. Rothenfluh, M. Abodeely, B. Kloss and M. W. Young (1998). *double-time* is a novel *Drosophila* clock gene that regulates PERIOD protein accumulation. *Cell* **94**: 83-95.

Quinn, W. G., P. P. Sziber and R. Booker (1979). The *Drosophila* memory mutant *amnesiac*. *Nature* **277**: 212-214.

Reginato, M. J., S. L. Krakow, S. T. Bailey and M. A. Lazar (1998). Prostaglandins promote and block adipogenesis through opposing effects on peroxisome proliferator-activated receptor gamma. *J. Biol. Chem.* **273**: 1855-1858.

Reisert, J., J. Lai, K. W. Yau and J. Bradley (2005). Mechanism of the excitatory Cl- response in mouse olfactory receptor neurons. *Neuron* **45**: 553-561.

Ren, D., B. Navarro, H. Xu, L. Yue, Q. Shi and D. E. Clapham (2001). A prokaryotic voltage-gated sodium channel. *Science* **294**: 2372-2375.

Renn, S. C., J. H. Park, M. Rosbash, J. C. Hall and P. H. Taghert (1999). A pdf neuropeptide gene mutation and ablation of PDF neurons each cause severe abnormalities of behavioral circadian rhythms in *Drosophila*. *Cell* **99**: 791-802.

Restrepo, D., J. H. Teeter and D. Schild (1996). Second messenger signaling in olfactory transduction. *J. Neurobiol.* **30**: 37-48.

Riddle, D. R., L. D. Wong and B. Oakley (1993). Lectin identification of olfactory receptor neuron subclasses with segregated central projections. *J. Neurosci.* **13**: 3018-3033.

Riffell, J. A., H. Lei, T. A. Christensen and J. G. Hildebrand (2009). Characterization and coding of behaviorally significant odor mixtures. *Curr. Biol.* **19**: 335-340.

Robertson, H. M., C. R. Preston, R. W. Phillis, D. M. Johnson-Schlitz, W. K. Benz and W. R. Engels (1988). A stable genomic source of P element transposase in *Drosophila melanogaster*. *Genetics* **118**: 461-470.

Robertson, H. M., C. G. Warr and J. R. Carlson (2003). Molecular evolution of the insect chemoreceptor gene superfamily in *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. U S A* **100 Suppl 2**: 14537-14542.

Rodan, A. R., J. A. Kiger, Jr. and U. Heberlein (2002). Functional dissection of neuroanatomical loci regulating ethanol sensitivity in *Drosophila*. *J. Neurosci.* **22**: 9490-9501.

Roller, L., Y. Tanaka and S. Tanaka (2003). Corazonin and corazonin-like substances in the central nervous system of the Pterygote and Apterygote insects. *Cell Tissue Res.* **312**: 393-406.

Ronnett, G. V. and C. Moon (2002). G proteins and olfactory signal transduction. *Annu. Rev. Physiol.* **64**: 189-222.

Rosbash, M., R. Allada, M. Dembinska, W. Q. Guo, M. Le, S. Marrus, Z. Qian, J. Rutila, J. Yaglom and H. Zeng (1996). A *Drosophila* circadian clock. *Cold Spring Harb. Symp. Quant. Biol.* **61**: 265-278.

Rothenfluh, A., M. Abodeely, J. L. Price and M. W. Young (2000a). Isolation and analysis of six timeless alleles that cause short- or long-period circadian rhythms in *Drosophila*. *Genetics* **156**: 665-675.

Rothenfluh, A., M. Abodeely and M. W. Young (2000b). Short-period mutations of per affect a double-time-dependent step in the *Drosophila* circadian clock. *Curr. Biol.* **10**: 1399-1402.

Rothenfluh, A., M. W. Young and L. Saez (2000c). A TIMELESS-independent function for PERIOD proteins in the *Drosophila* clock. *Neuron* **26**: 505-514.

Rouquier, S., C. Friedman, C. Delettre, G. van den Engh, A. Blancher, B. Crouau-Roy, B. J. Trask and D. Giorgi (1998). A gene recently inactivated in human defines a new olfactory receptor family in mammals. *Hum. Mol. Genet.* **7**: 1337-1345.

Rubin, G. M. and A. C. Spradling (1982). Genetic transformation of *Drosophila* with transposable element vectors. *Science* **218**: 348-353.

Sandman, C. A., P. Wadhwa, L. Glynn, A. Chicz-Demet, M. Porto and T. J. Garite (1999). Corticotrophin-releasing hormone and fetal responses in human pregnancy. *Ann. N Y Acad. Sci*. **897**: 66-75.

Sato, K., M. Pellegrino, T. Nakagawa, L. B. Vosshall and K. Touhara (2008). Insect olfactory receptors are heteromeric ligand-gated ion channels. *Nature* **452**: 1002-1006.

Schneider, L. E., M. S. Roberts and P. H. Taghert (1993). Cell type-specific transcriptional regulation of the *Drosophila* FMRFamide neuropeptide gene." *Neuron* **10**: 279-291.

Scholz, H., J. Ramond, C. M. Singh and U. Heberlein (2000). Functional ethanol tolerance in *Drosophila*. *Neuron* **28**: 261-271.

Schulz, R. A., C. Chromey, M. F. Lu, B. Zhao and E. N. Olson (1996). Expression of the D-MEF2 transcription in the *Drosophila* brain suggests a role in neuronal cell differentiation. *Oncogene* **12**: 1827-1831.

Schwartz, W. J. (2004). Sunrise and sunset in fly brains. *Nature* **431**: 751-752.

Selbie, L. A., A. Townsend-Nicholson, T. P. Iismaa and J. Shine (1992). Novel G protein-coupled receptors: a gene family of putative human olfactory receptor sequences. *Brain Res. Mol. Brain Res.* **13**: 159-163.

Sharma, Y., U. Cheung, E. W. Larsen and D. F. Eberl (2002). *pPTGAL*, a convenient *Gal4 P*-element vector for testing expression of enhancer fragments in *Drosophila*. *Genesis* **34**: 115-8.

Shaw, P. J., N. S. Wratten, A. P. McGregor and G. A. Dover (2002). Coevolution in bicoid-dependent promoters and the inception of regulatory incompatibilities among species of higher Diptera. *Evol. Dev.* **4**: 265-277.

Shearman, L. P., X. Jin, C. Lee, S. M. Reppert and D. R. Weaver (2000a). Targeted disruption of the *mPer3* gene: subtle effects on circadian clock function. *Mol. Cell. **Biol*. 20: 6269-6275.**

Shearman, L. P., S. Sriram, D. R. Weaver, E. S. Maywood, I. Chaves, B. Zheng, K. Kume, C. C. Lee, G. T. van der Horst, M. H. Hastings and S. M. Reppert (2000b). Interacting molecular loops in the mammalian circadian clock. *Science* **288**: 1013-1039.

Shehata, A. M., E. M. Mark and H. J. Phaff (1955). Yeasts isolated from *Drosophila* and from their suspected feeding places in southern and central California." *Mycologia* **47**: 799-811.

Shen, P. and H. N. Cai (2001). *Drosophila* neuropeptide F mediates integration of chemosensory stimulation and conditioning of the nervous system by food. *J. Neurobiol.* **47**: 16-25.

Shiga, S., N. T. Davis and J. G. Hildebrand (2003). Role of neurosecretory cells in the photoperiodic induction of pupal diapause of the tobacco hornworm *Manduca sexta*. *J. Comp. Neurol.* **462**: 275-285.

Shiga, S. and H. Numata (2000). The role of neurosecretory neurons in the pars intercerebralis and pars lateralis in reproductive diapause of the blowfly, *Protophormia terraenovae*. *Naturwissenschaften* **87**: 125-128.

Shiomi, K., Y. Fujiwara, T. Atsumi, Z. Kajiura, M. Nakagaki, Y. Tanaka, A. Mizoguchi, T. Yaginuma and O. Yamashita (2005). Myocyte enhancer factor 2 (MEF2) is a key modulator of the expression of the prothoracicotropic hormone gene in the silkworm, *Bombyx mori*. *Febs J.* **272**: 3853-3862.

Smale, S. T. (1997). Transcription initiation from TATA-less promoters within eukaryotic protein-coding genes. *Biochim. Biophys. Acta.* **1351**: 73-88.

So, W. V. and M. Rosbash (1997). Post-transcriptional regulation contributes to *Drosophila* clock gene mRNA cycling. *EMBO J***. 16**(23): 7146-7155.

Song, H. J., J. C. Billeter, E. Reynaud, T. Carlo, E. P. Spana, N. Perrimon, S. F. Goodwin, B. S. Baker and B. J. Taylor (2002). The fruitless gene is required for the proper formation of axonal tracts in the embryonic central nervous system of *Drosophila*. *Genetics* **162**: 1703-1724.

Spletter, M. L. and L. Luo (2009). A new family of odorant receptors in *Drosophila*." *Cell* **136**: 23-25.

Sprince, H., C. M. Parker, G. G. Smith and L. J. Gonzales (1974). Protection against acetaldehyde toxicity in the rat by L-cysteine, thiamin and L-2-methylthiazolidine-4-carboxylic acid. *Agents Actions* **4**: 125-130.

Stanek, D. M., J. Pohl, J. W. Crim and M. R. Brown (2002). Neuropeptide F and its expression in the yellow fever mosquito, *Aedes aegypti*. *Peptides* **23**: 1367-1378.

Stocker, R. F. (1994). The organization of the chemosensory system in *Drosophila melanogaster*: a review. *Cell Tissue Res.* **275**: 3-26.

Stortkuhl, K. F., A. Hofbauer, V. Keller, N. Gendre and R. F. Stocker (1994). Analysis of immunocytochemical staining patterns in the antennal system of *Drosophila melanogaster*. *Cell Tissue Res.* **275**: 27-38.

Strand, F. L. (1999). Neuropeptides: Regulators of Physiological Processes Cellular and Molecular Neuroscience Series. *Cambrige, Mass.MIT Press.*

Tabakoff, B. and P. L. Hoffman (1996). Alcohol addiction: an enigma among us. *Neuron* **16**: 909-912.

Taghert, P. H. and L. E. Schneider (1990). Interspecific comparison of a *Drosophila* gene encoding FMRFamide-related neuropeptides. *J. Neurosci.* **10**: 1929-1942.

Tamaru, T., Y. Isojima, T. Yamada, M. Okada, K. Nagai and K. Takamatsu (2000). Light and glutamate-induced degradation of the circadian oscillating protein BMAL1 during the mammalian clock resetting. *J. Neurosci.* **20**: 7525-7530.

Tamura, K., S. Subramanian and S. Kumar (2004). Temporal patterns of fruit fly (*Drosophila*) evolution revealed by mutation clocks. *Mol. Biol. Evol.* **21**: 36-44.

Tanaka, S. (2001). Endocrine mechanisms controlling body-color polymorphism in locusts. *Arch. Insect Biochem. Physiol.* **47**: 139-149.

Tanaka, Y., Y. Hua, L. Roller and S. Tanaka (2002). Corazonin reduces the spinning rate in the silkworm, *Bombyx mori*. *J. Insect. Physiol.* **48:** 707-714.

Tank, A. W. and H. Weiner (1979). Ethanol-induced alteration of dopamine metabolism in rat liver. *Biochem. Pharmacol.* **28**: 3139-3147.

Tawfik, A. I., S. Tanaka, A. De Loof, L. Schoofs, G. Baggerman, E. Waelkens, R. Derua, Y. Milner, Y. Yerushalmi and M. P. Pener (1999). Identification of the

gregarization-associated dark-pigmentotropin in locusts through an albino mutant. *Proc. Natl. Acad. Sci. U S A* **96**: 7083-7087.

Thomasson, H. R., J. D. Beard and T. K. Li (1995). ADH2 gene polymorphisms are determinants of alcohol pharmacokinetics. *Alcohol Clin. Exp. Res.* **19**: 1494-1499.

Urizar, N. L., Z. Yang, H. J. Edenberg and R. L. Davis (2007). *Drosophila* homer is required in a small set of neurons including the ellipsoid body for normal ethanol sensitivity and tolerance*. J. Neurosci.* **27**: 4541-4551.

Vassar, R., J. Ngai and R. Axel (1993). Spatial segregation of odorant receptor expression in the mammalian olfactory epithelium. *Cell* **74**: 309-318.

Veenstra, J. A. (1989). Isolation and structure of corazonin, a cardioactive peptide from the American cockroach. *FEBS Lett* **250**: 231-234.

Veenstra, J. A. (1991). Presence of corazonin in three insect species, and isolation and identification of [His^{7}]corazonin from *Schistocerca americana*. *Peptides* **12**: 1285-1289.

Veenstra, J. A. (1994). Isolation and structure of the *Drosophila* corazonin gene. *Biochem. Biophys. Res. Commun.* **204**: 292-296.

Venter, J. C., and Others (2001). The sequence of the human genome. *Science* **291**: 1304-1351.

Verleyen, P., G. Baggerman, I. Mertens, T. Vandersmissen, J. Huybrechts, A. Van Lommel, A. De Loof and L. Schoofs (2006). Cloning and characterization of a third isoform of corazonin in the honey bee *Apis mellifera*. *Peptides* **27**: 493-499.

Vitaterna, M. H., D. P. King, A. M. Chang, J. M. Kornhauser, P. L. Lowrey, J. D. McDonald, W. F. Dove, L. H. Pinto, F. W. Turek and J. S. Takahashi (1994).

Mutagenesis and mapping of a mouse gene, Clock, essential for circadian behavior. *Science* **264**: 719-725.

Vitaterna, M. H., C. H. Ko, A. M. Chang, E. D. Buhr, E. M. Fruechte, A. Schook, M. P. Antoch, F. W. Turek and J. S. Takahashi (2006). The mouse Clock mutation reduces circadian pacemaker amplitude and enhances efficacy of resetting stimuli and phase-response curve amplitude. *Proc. Natl. Acad. Sci. U S A* **103**: 9327-9332.

Vosshall, L. B. and R. F. Stocker (2007). Molecular architecture of smell and taste in *Drosophila*. *Annu. Rev. Neurosci.* **30**: 505-533.

Wand, G., M. Levine, L. Zweifel, W. Schwindinger and T. Abel (2001). The cAMP-protein kinase A signal transduction pathway modulates ethanol consumption and sedative effects of ethanol. *J. Neurosci.* **21**: 5297-5303.

Warr, C., P. Clyne, M. de Bruyne, J. Kim and J. R. Carlson (2001). Olfaction in *Drosophila*: coding, genetics and e-genetics. *Chem. Senses.* **26**: 201-206.

Wei, Q., Y. Rong and B. M. Paterson (2007). Stereotypic founder cell patterning and embryonic muscle formation in *Drosophila* require *nautilus* (MyoD) gene function. *Proc. Natl. Acad. Sci. U S A* **104**: 5461-5466.

Weiner, H. (1982). Aldehyde dehydrogenase. *Prog. Clin. Biol. Res.* **114**: 1-9.

Weiner, H. (1987). Subcellular localization of acetaldehyde oxidation in liver. *Ann. N Y Acad. Sci.* **492**: 25-34.

Weisinger, G. (1995). The transcriptional regulation of the preproenkephalin gene. *Biochem. J.* **307**: 617-629.

Wen, T., C. A. Parrish, D. Xu, Q. Wu and P. Shen (2005). *Drosophila* neuropeptide F and its receptor, NPFR1, define a signaling pathway that acutely modulates alcohol sensitivity. *Proc. Natl. Acad. Sci. U S A* **102**: 2141-2146.

Wentworth, B. M., M. Donoghue, J. C. Engert, E. B. Berglund and N. Rosenthal (1991). Paired MyoD-binding sites regulate myosin light chain gene expression. *Proc. Natl. Acad. Sci. U S A* **88**: 1242-1246.

Weiss, F. and L. J. Porrino (2002). Behavioral neurobiology of alcohol addiction: recent advances and challenges. *J. Neurosci.* **22**: 3332-3337.

White, B. H., T. P. Osterwalder, K. S. Yoon, W. J. Joiner, M. D. Whim, L. K. Kaczmarek and H. Keshishian (2001). Targeted attenuation of electrical activity in *Drosophila* using a genetically modified K(+) channel. *Neuron* **31**: 699-711.

White, J., K. A. Hamilton, S. R. Neff and J. S. Kauer (1992). Emergent properties of odor information coding in a representational model of the salamander olfactory bulb. *J. Neurosci.* **12**: 1772-1780.

Wicher, D., R. Schafer, R. Bauernfeind, M. C. Stensmyr, R. Heller, S. H. Heinemann and B. S. Hansson (2008). *Drosophila* odorant receptors are both ligand-gated and cyclic-nucleotide-activated cation channels. *Nature* **452**: 1007-1011.

Wilson, R. I. and G. Laurent (2005). Role of GABAergic inhibition in shaping odor-evoked spatiotemporal patterns in the *Drosophila* antennal lobe. *J. Neurosci.* **25**: 9069-9079.

Wistrand, M., L. Kall and E. L. Sonnhammer (2006). A general model of G protein-coupled receptor sequences and its application to detect remote homologs. *Protein Sci***. 15**: 509-521.

Wittkopp, P. J., B. K. Haerum and A. G. Clark (2006). Parent-of-origin effects on mRNA expression in *Drosophila melanogaster* not caused by genomic imprinting. *Genetics* **173**(3): 1817-1821.

Wolf, F. W. and U. Heberlein (2003). Invertebrate models of drug abuse. *J. Neurobiol.* **54**: 161-178.

Woodard, C., T. Huang, H. Sun, S. L. Helfand and J. Carlson (1989). Genetic analysis of olfactory behavior in *Drosophila*: a new screen yields the ota mutants. *Genetics* **123**: 315-326.

Wu, Q., T. Wen, G. Lee, J. H. Park, H. N. Cai and P. Shen (2003). Developmental control of foraging and social behavior by the *Drosophila* neuropeptide Y-like system. *Neuron* **39**: 147-161.

Yagita, K., F. Tamanini, G. T. van Der Horst and H. Okamura (2001). Molecular mechanisms of the biological clock in cultured fibroblasts. *Science* **292**: 278-281.

Yamamori, E., M. Asai, M. Yoshida, K. Takano, K. Itoi, Y. Oiso and Y. Iwasaki (2004). Calcium/calmodulin kinase IV pathway is involved in the transcriptional regulation of the corticotropin-releasing hormone gene promoter in neuronal cells. *J. Mol. Endocrinol.* **33**: 639-649.

Yerushalmi, Y., K. Bhargava, C. Gilon and M. P. Pener (2002). Structure-activity relations of the dark-colour-inducing neurohormone of locusts. *Insect Biochem. Mol. Biol.* **32**: 909-917.

Yoshida, A., I. Y. Huang and M. Ikawa (1984). Molecular abnormality of an inactive aldehyde dehydrogenase variant commonly found in Orientals. *Proc. Natl. Acad. Sci. U S A* **81**: 258-261.

Zakhari, S. (2006). Overview: how is alcohol metabolized by the body? *Alcohol Res. Health* **29**: 245-254.

Zhang, Z. and M. Gerstein (2003). Of mice and men: phylogenetic footprinting aids the discovery of regulatory elements. *J. Biol.* **2**: 11.

Zheng, B., U. Albrecht, K. Kaasik, M. Sage, W. Lu, S. Vaishnav, Q. Li, Z. S. Sun, G. Eichele, A. Bradley and C. C. Lee (2001). Nonredundant roles of the *mPer1* and *mPer2* genes in the mammalian circadian clock. *Cell* **105**: 683-694.

Zheng, B., D. W. Larkin, U. Albrecht, Z. S. Sun, M. Sage, G. Eichele, C. C. Lee and A. Bradley (1999). The *mPer2* gene encodes a functional component of the mammalian circadian clock. *Nature* **400**: 169-173.

Zheng, J., R. Mutcherson, 2nd and S. L. Helfand (2005). Calorie restriction delays lipid oxidative damage in *Drosophila melanogaster*. *Aging Cell* **4**: 209-216.

Zozulya, S., F. Echeverri and T. Nguyen (2001). The human olfactory receptor repertoire. *Genome Biol.* **2**: RESEARCH0018.

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