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The Roles of *Hox* Genes in the Corazonergic Neurons of Neuroblast7-3 Sublineage

신경아세포 7-3 계보인 Corazonin 신경에서 *Hox* 유전자의 역할

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The Roles of *Hox* Genes in the Corazonergic Neurons of Neuroblast7-3 Sublineage

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ABSTRACT

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In *Drosophila* development, *Hox* genes determine the cell fate in a segment specific manner. Recent studies have shown that *Hox* genes have a part in causing programmed cell death in neurogenesis at the level of neuroectoderm. This study shed lights on the roles of *Hox* genes in the NB7-3 sublineages particularly in corazonergic neuronal cell fate specification and apoptosis. By observation of the *Ubx* and *abd-A* mutant, these two genes are promoting factor for corazonergic lineage. On the other hand, the overexpression of *Abd-B* which endogenously expressed in posterior region, PS10-14 caused the lack of corazonin neuron in anterior segment, while extra corazonin neurons in A7-A8 segments were detected in *Abd-B* loss-of-function mutant. This region is identical to the rescued cell when the programmed cell death was inhibited. This data suggest that *Abd-B* exerts the pro-apoptotic role in NB7-3 development during embryogenesis. To figure out the effect of altered corazonin neurons caused by *Hox* gene mutation, we additionally examined the ethanol sedation assay in adult flies. Along the level of corazonin expression, known as a stress regulator, the resistance to the ethanol induced sedation was distinct to sex. Specially, male showed more significant differences than female in the respect of corazonin expression.

Keywords : Neuroblast 7-3, Corazonin, *Hox* gene, Ethanol response, *Drosophila melanogastor*

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CHAPTER 1. INTRODUCTION

1.1. Study Background

In *Drosophila melanogaster*, the nervous system consists of the brain and ventral nerve cord (VNC) derived from the neuroblasts (NBs), which differentiates to neuron and glia (Novotny et al., 2002). This neurogenesis is initiated with the delamination of neuroblasts from neuro ectoderm per hemi segment (Bossing et al., 1996), and it has been maintained evenly under the epidermis (Bate, 1976; Bate and Grunewald, 1981; Goodman et al., 1984; Doe and Goodman, 1985; Schimidt, 1997). In every parasegment (PS), there are 30 embryonic neuroblasts (NBs) including 13 dorsal neuroblasts and 17 ventral neuroblasts. They are divided asymmetrically into smaller ganglion mother cells (GMCs) (Doe, 1992), forming about 350 neurons and 30 glial cells during embryogenesis. Especially, the ventral ganglia of the 13 NBs located in dorsal side produce 22-27 glial cells, forming the 90% of total glial cells (Schimidt, 1997). All cell types of embryonic central nervous system, interneuron, glial cells, motor neuron and neural secretory cells are derived from neuroblasts. The similar dorso-ventral region's NBs are developed from the similar lineages and produce the same motor neuronal subtype. Among them, the NB7-3, the serotonergic lineage has been usually studied because NB7-3 is easy to identify by using spatiotemporal molecular markers and to figure out its relatively small number of neuronal precursors.

Generally, NB7-3 generates only 3 ganglion mother cells (GMCs), GMC1, GMC2 and GMC3. At first, GMC1 is differentiated to the serotonergic neuron (EW1) and the motor neuron (GW) which goes through apoptosis caused by notch signaling pathway (Lundell et al., 2003). Secondly, GMC2 makes the other serotonergic neuron and its sibling, going to apoptosis by notch signaling pathway like GW neuron. Lastly, GMC3 becomes EW3, which produces neuropeptide corazonin, and corazonin expression

accompanies neuronal differentiation and is maintained steady highly in differentiated neurons (Choi et al., 2005).

In recent studies, the segment specific identity and its pattern are caused by *Hox* genes. *Hox* genes are composed of the *Antennapedia complex* (ANT-C), controlling head, gnathal and anterior thoracic segment, and bithorax complex (BX-C), shaping the posterior thoracic and all abdominal regions in *Drosophila.* Notably, *Hox* genes have multiple roles in segmental patterning in CNS. In early NB, *Hox* genes controlled the different cell specification, and well known corazonin expression in ventral nerve cord is limited to the thoracic and abdominal region.

Besides, there are segment specific differences in NB7-3 development, remarkably in the C1-T1 and A7-A8 segments. It has been shown that segments' fate of the NB7-3 were changed by *Ubx* and *abd-A* in BX-C (Technau, 2013). Also, NBs in the tail region (A8-A11) of abdominal segments contain less neuromers, which is assumed *Abd-B* has a major effect on the cell death in the posterior portion of the VNC. In brief, *Hox* genes have major roles in the cell type specification and programmed cell death during neurogenesis (Reichert and Bello, 2010; Rogulja-Ortmann and Technau, 2008).

1.2. Purpose of Research

Hox genes are involved in the cell fate determination and programmed cell death. There are the variability of expression in C1 to T1 and A7 to A8 when *Hox* genes are manipulated, and the cell fates and serial differentiation only in post-embryonic stage has been figured out. Besides, the null mutation of *Hox* gene is embryonic lethal so it is hard to examine the role of each *Hox* gene in NB7-3 development.

The aim of this study is to confirm and extend previous work on the thoracic and abdominal region of the corazonin neuron, one of the NB7-3 sublineage. This study enables to know whether the *Hox* genes are related to the root of neuronal variation and

maintenance by focusing on the minute research in embryogenesis. In this study, three *Hox* genes, *Ultrabithorax (Ubx), Abdominal-A (abd-A) and Abdominal-B (Abd-B)* were chosen to understand how *Hox* gene acts on maintaining the corazonergic neuron in developmental stages.

Moreover, the alteration of neuropeptide corazonin level may also affect its function. According to Yan Zhao (2010), Corazonin neurons are essential for normal stress-induced behavior and the sex-specific alcohol response in *Drosophila* (Devineni and Heberlein, 2012; Mclure and Heberlein, 2013), but the sex-dimorphic ethanol-resistance in drosophila has not been studied yet.

Functional analysis would be helpful to understand the neuropeptide corazonin function in different corazonin expression.

CHAPTER 2. MATERIALS AND METHODS

2.1. Fly Strains

The following fly lines and mutant stocks were used. Flies are raised at 25℃ in food vials. All Oregon-R (OR), Canton-S (CS) and w^{1118} were used as wild type. All control strains were tested, including Oregon-R and Canton-S for functional analysis.

To analyze the effects of gain-of-function, the GAL4/UAS system was used. The UAS lines were crossed to *eg-GAL4 (Mz360)* and *crz-GAL4* (Choi et al., 2005) drivers to make ectopic overexpression of the *Hox* genes specifically in the NB7-3 sublineages. *w; UAS-Abd-B, w; UAS- abd-A,* and *w; UAS-Ubx* (Bloomington stock center).

As the loss of function mutants, the deficiency lines of bithorax complex component of *Hox* genes were used: *Ubx, abd-A, Abd-B, Df(3R)ubx109/Dp3;3(p5)*, double mutation of *abd-A* and *Ubx.* A *UAS-p35* was used (Hay et al., 1994) to block the upstream of programmed cell death pathway in the entire NB7-3 lineage.

2.2. Whole Mount Immunohistochemistry of Larval CNS

Whole-mount immunohistochemistry in larval CNS was performed after dissection. The dissected 3rd instar larvae tissues were fixed in 4% paraformaldehyde about 1hr at room temperature (RT). After washing 6 times with PT solution 10mins each, the samples were pre-incubated for 0.5~2hr in the 5% NGS (DSHB) blocking solution. The 1st anti-rabbit corazonin antibody (A gift from Dr. Gyung Hee Lee) was applied directly to the samples for overnight at 4℃, and put the samples at room temperature for 0.5hr. Then they were rinsed in PT 6 times 10 mins each at room temperature. The fluorescent secondary antibody (Alexa Fluoro

488 goat anti-rabbit IgG (H+L), Invitrogen) was applied (1:1000 dilution) in PT for 0.5~1hr at RT. The samples were washed 6 times for 15 mins each and replaced with mounting solution.

2.3. Dissection of Live Embryo

The late embryo's (17-22hr AEL, 25℃) cuticle is thickened, which impedes the penetration of antibody. Moreover, some antibody itself is hard to penetrate the cuticle. Corazonin is begun to express late embryo stage and it is so minute neuropeptide that hard to be detected (Figure 1E). Therefore, the whole mount preparation of late embryo was needed to be dissected, making them as an embryo fillet. All procedures were preceded by the IHC method which was described above, allowing the immunosignal to detect easier.

The embryos between 14-17 stages were firstly collected for 2-3hr at 25℃ by using egg collection plate, and incubated for <20hr at 19℃, so that they would be at stage 15-16. Staging also can be done by associating gut morphology. Since the body wall at st 16-17 was hard to stick onto the glass slide, the ideal state of dissection was when the gut has divided into 3 parts-foregut, midgut, and hindgut.

For embryos dechorionation, the double-sided tapes were stuck on to the slide and transferred the desired age embryo eggs to the taped slide. After gently rolling the egg with blunt end forceps, the dechorionated embryos were aligned on agar. Then, the aligned embryos were lifted off from the cut agar to the other double sided taped slide (Figure 1A). At this slide, the clear nail polish was applied 30-40mm long to make a dissection chamber so that there is 1mm of space between the chamber margin and tape. Then, the chamber was filled with 1X PBS (Figure 1B).

By using tungsten dissection needle, the posterior end of each embryo was stabbed and cut along the dorsal midline to remove a vitelline membrane (Figure 1C). While the embryos were kept in buffer, a small cut along the dorsal midline were made and the body

wall was separated to stick on the slide not to move, and then guts were removed (Figure 1D).

After the embryos were dissected, these embryo fillets were fixed in 4% paraformaldehyde about 30-45 minutes at room temperature. By using two pasteur pipettes, the samples were washed gently with 1ml of 1xPBS for 6 times and 1ml of 1xPBT. After washing, the 5% NGS was applied in 1xPBT to block non-specific staining over 45 minutes. The rest of blocking solution was wiped to apply 1st antibody directly. The subsequent steps were similar to general immunostaining method mentioned above.

2.4. Ethanol Sedation Assay

Commonly, there were three measurement for alcohol sensitivity - analyze the time that 50% of flies being LoRR (loss of righting reflex), monitoring locomotion activity. To measure ethanol induced sedation, in this study, SSA (Simple Sedation Assay) was performed. All flies were collected the first day after eclosion to experiment with healthy ones. The collected flies were exposed to vaporous high concentration of ethanol (85% ethanol). The sedation sensitivity was quantified as the sedation time 50 (ST50), which meant the time that 50% of flies were sedated. Therefore, higher ST50 meant that the flies had more resistance to ethanol.

Since the day before performing the assay, 11 adult flies separated by sex had been collected in one vial with 1-5 minutes anesthesia. 85% ethanol solution appropriately diluted from 100% ethanol was prepared at RT. They were incubated for 24hr in constant condition with 12:12LD cycle at 25℃. After transferring adult flies without anesthesia to empty food vial (No food in vial), the vial was sealed with cotton plug. The ethanol solution was applied on the cotton plug directly and the solution applied vial was covered with paraffin film sealed cotton plug (Figure 2). From the cotton plug, vaporized ethanol was provided, causing progressive increases internal ethanol and sedation. During the test, the 4 vials were prepared with 1 minute interval, and every 6 minutes the vials

were gently tapped 3 times and then the number of flies immobile or death were counted. Time dependently, the flies standing in the absence of walking, lying on their backs with or without flapping wings were counted. The testing room was 20–25℃ and all experiments were performed in anonymous code to prevent unintended bias.

2.5. Statistical Analysis

Three replicated vials of 11 flies in each group were measured. The statistical analysis was performed by using GraphPad prism 6.0. All graph show mean with SEM.

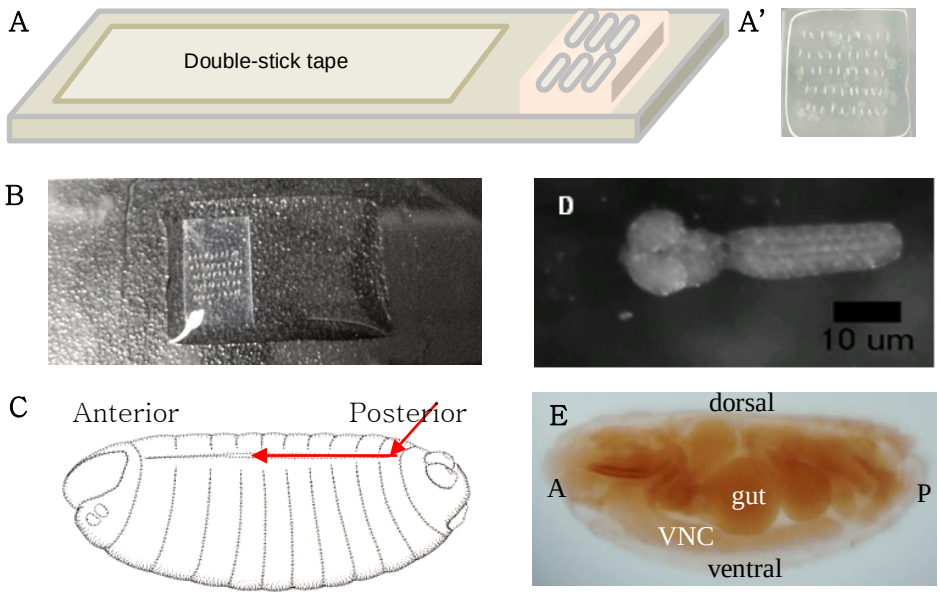


Figure 1. The method of live embryo dissection Since embryonic cuticle is accumulated in late stage of embryogenesis, embryo should be dissected before immunostaining. (A) To remove chorion, roll the embryos on double-stick tape. A' shows the aligned embryos on agar after dechorionation. (B) The 2nd slide with dissection chamber. (C) The arrows indicate the direction of dissection to remove vitelline membrane. (D) Dissected embryo fillet, exposing the embryonic CNS. (E) The late embryo stained with general IHC method. The corazonin was hard to detect in late embryo without dissection.

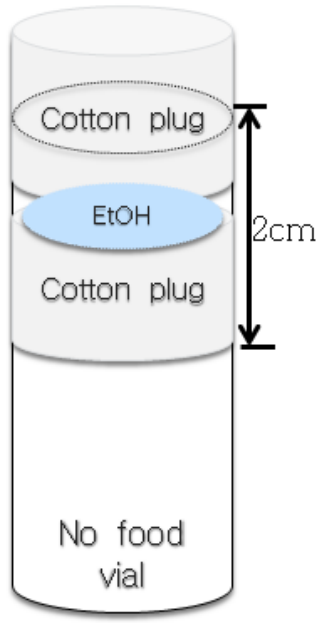


Figure 2. The modified equipment for the simple sedation assay (SSA) Three replicated vials of 11 flies in each group were measured. The ethanol solution was applied on the cotton plug directly and the solution applied vial was covered with paraffin film sealed cotton plug.

CHAPTER 3. RESULTS

3.1. Corazonin Neurons in the *Drosophila* Central Nervous System

The peak expression of corazonin was in 18-24 hour after egg laying (AEL) and in the following post-embryonic stage. During embryogenesis, the most apparent immunofluorescent expression of corazonin is at stage 16. The later stage embryo was, the higher level of corazonin was detected in ventral nerve cord (VNC). In both Oregon-R and Canton-S, the immunoreactivity was detected in the 8 pairs of corazonergic neurons of VNC in T2-A6 segments (Figures 3A-C). This embryonic corazonin expression is the same as larvae. The corazonin expression was regulated temporally in embryo. Since the corazonin neural cells in medial segments begin to appear faintly at first and also showed higher immunoreactivity in later stages (Figure 3D). That is, corazonin shows spatio-temporal differences, and corazonin was firstly accumulated in the more medial segments.

Hence, the wild type corazonin expression had been formed in T2-A6 at stage 15-16 and maintained before undergoing metamorphosis.

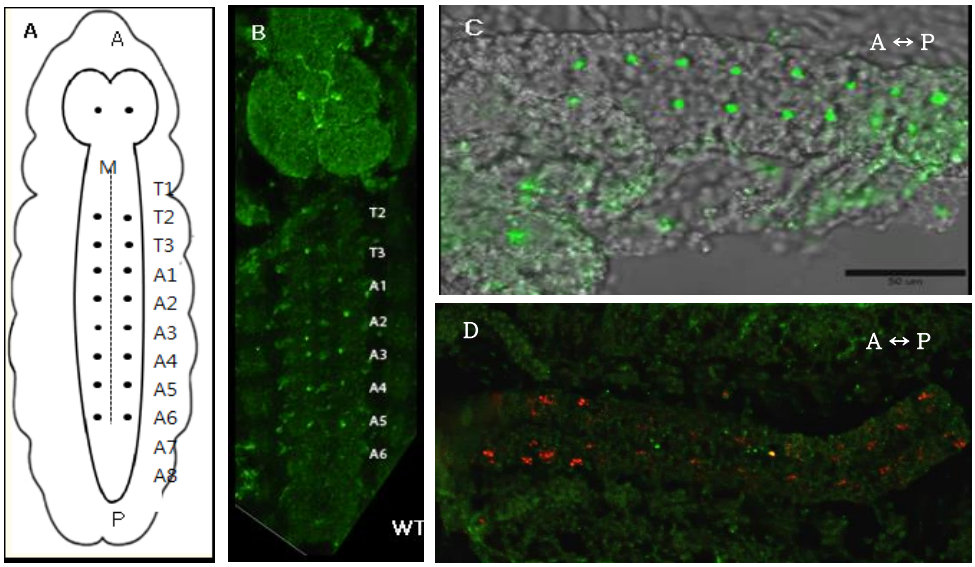


Figure 3. The expression of corazonin in wild type (A–C) Three strains (Oregon–R, Canton–S and WT crossed with *eg–GAL4*) were dissected and immunostained as control. Each embryo is dissected at stage 16, and the embryonic stages were determined by their morphology and the relatively estimated time after egg laying (Campos–Ortega and Hartenstein, 1985). (A) Diagram of segment specific expression of coraznonin neuron in central nervous system derived from Neuroblast 7–3 (A: Anterior, P: Posterior). (B) Canton–S expression which is maximized deconvolution image. (C) The brain lobes and ventral nerve cord expression of Oregon–R (overlay with the separated intensity of DIC image) (D) Double staining of corazonin and *eg* (general NB7–3 marker) at stage 14. Only *eg* (red staining) has immunoreactivity, which indicates the temporal development of corazonin neurons in the *Drosophila* CNS.

3.2. *Ubx* and *Abd-A* Promote Corazonergic Sub-lineage

Hox genes specify the cell fate and cause programmed cell death in a segment-specific manner. As one of the bithorax complex *Hox* gene, *Ubx* is endogenously expressed at the anteriormost part of bithorax complex, which is shown in para segment 5-13, with strongest expression in posterior T3 and anterior A1 and *abd-A* is expressed in posterior A1 to anterior A7 (Beachy et al., 1985; Carroll et al., 1988; White and Wilcox, 1985).

Since the spatial distribution of corazonin neuron in wild type overlapped with the endogenous expression of *Ubx* and *abd-A,* I hypothesized that these two genes might be a promoting factor for conforming corazonin neurons.

To figure out their segment specific manner, overexpression phenotypes were studied at first. The ectopic expression of *Ubx* driven by *eg-GAL4* showed normally expressed in T2-A6, but sometimes its expression was extended to T1 (Figure 4B). In 3rd instar larvae, however, *Ubx* overexpression was observed in various. In frequent, the extra corazonin neurons were detected in C3-T1, as well as in more anterior gnathal segments C1-C2. The second most frequent phenotype is devoid of hemisegment in T2-A6. Since *Ubx* overexpression is variable that it is insufficient to predict the role. Moreover, the gain-of-function mutations of *Hox* genes transform posterior structures into copies of anterior ones, it is necessary to confirm null mutant phenotype, adding to homeotic transformation.

Remarkably, the corazonin expression in null mutants of *Hox* gene supports the results from the gain-of-function mutations. In *Ubx* null mutant, only 6 or 7 pairs of vCrz were expressed specially in the segment A1-A6 or A2-A6 (Figure 5B). That is, the T2 or both T2 and T3 neurons were missing. Since these regions are highly affected by *Ubx, Ubx* can be a factor which is promoting corazonergic sublineage.

Meanwhile, *eg-GAL4/UAS-abdA*, the CNS ectopically

overexpressed *abd-A*, the corazonin neurons were extended to 1 or 2 anterior segments as usual. Add to remained endogenous vCrz neurons in T2-A6, 8 or 9 pairs were usually observed (Figure 4C). Generally, *Ubx* expression is suppressed by *abd-A*, partially and strongly expressed in parasegment 6, but *Ubx* which has positive role in forming corazonin neuron is influential in *abd-A* mutant. Therefore it is also difficult to interpret *abd-A* function in separate. The reason is that posterior dominance of *Hox* genes, *abdominal-A (abd-A)* dominates over the more posterior *Abdominal-B (Abd-B)* and the more anterior *Ultrabithorax (Ubx)* (Capovilla and Botas, 1998) in usual.

Theoretically, *Df(3R)ubx109/Dp3;3(p5)*, double mutation of *Ubx* and *abd-A* specifically in PS5-13 is not controlled by both *Ubx* and *abd-A* so only *Abd-B* do the segment specific role in PS10-14 determination. As I mentioned above, *Ubx* and *abd-A* each showed agonistic role of producing corazonergic neurons. The loss of homeotic control in PS5-13, only one pair of corazonin neurons were observed in T2, the anterior-most segment of corazonin neurons. Or all vCRZ neurons were missing, remained intact only in the brain lobes (Figure 5D). These results reinforced both *Ubx* and *abd-A* had a major role in activating corazonergic neuron formation.

3.3. Downregulation of *Abd-B* in Posterior Corazonin Neurons

Naturally, *Abd-B* is endogenously expressed in PS 10-14, and it has been known that *Abd-B* prevents the forming tail region of some neuromeres in A9-10, backing the *Abd-B* induced programmed cell death in NB7-3 (Birkholz et al., 2013). As hypothesis of *Abd-B*'s role in absence of corzonergic neuron in A7-A8, the corazonergic neuronal expression in *Abd-B* loss-of-function was extended to rear part of embryo particularly in A7 and A8 so 10 pairs of vCrz neurons were observed (Figure 5E).

Furthermore, when *Abd-B* was ectopically overexpressed in 3rd larvae, the more anterior segments were controlled by *Abd-B* so there were completely depleted vCrz in about 25% samples (n=39).

Besides, UAS lines were crossed to *crz-GAL4* driver (Choi et al., 2005) to ectopically overexpress each of the *Hox* genes in the NB7-3 lineage, which performed in the same way in previous work. As a control, *crz-GAL4* showed immunoreactivity in T2-A6 just like WT phenotype. While both overexpression of *Ubx* and *abd-A* were not deformed vCrz expression pattern, ectopically overexpressed by *Abd-B* mainly showed none neural cells (17%) or 2 pairs missing so that only A2-A6 were detected (55%) in 3rd instar larvae. Therefore, the lack of corzonin neurons gave a reason to *Abd-B* triggered cell death or *Abd-B* involved in repression of corazonergic neural formation.

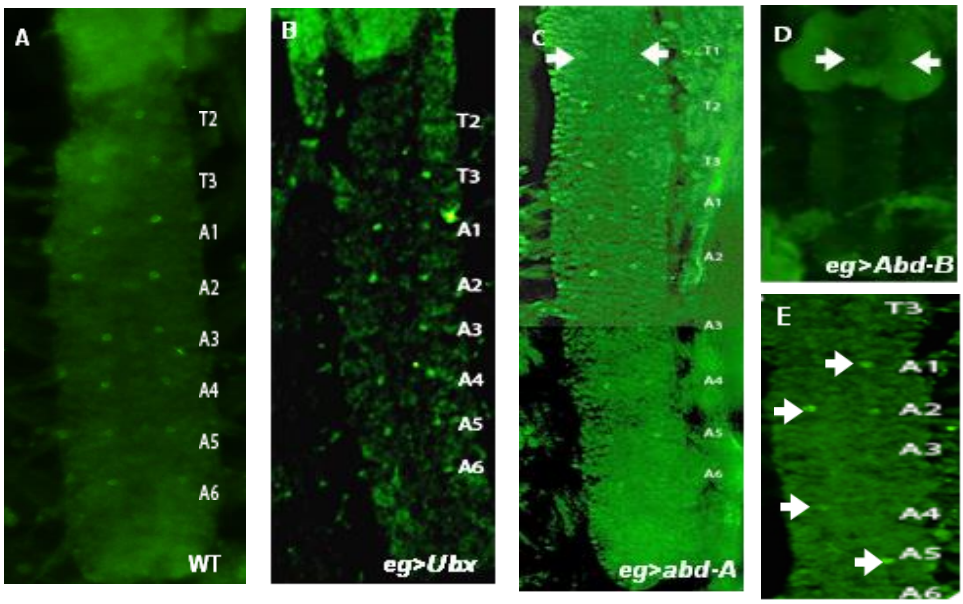


Figure 4. The effect of overexpressed *Hox* genes on forming corazonin neurons (A) Corazonin neurons were expressed in T2-A6 in wildtype. (B) The ectopically overexpressed *Ubx* mutant had similar expression compared to wild type. (C) The corazonin expression was extended to anterior segment, T1 in *eg-abd-A/UAS-abd-A.* (D-E) Corazonin neural cells were disappeared in *Abd-B* gain-of-function mutant. (D) Only corazonin neurons in brain remained (stage 17 embryo). (E) Numerous cells were missing in VNC, and the white arrows indicate the normally expressed corazonin neuron in *eg-GAL4/UAS-Abd-B.*

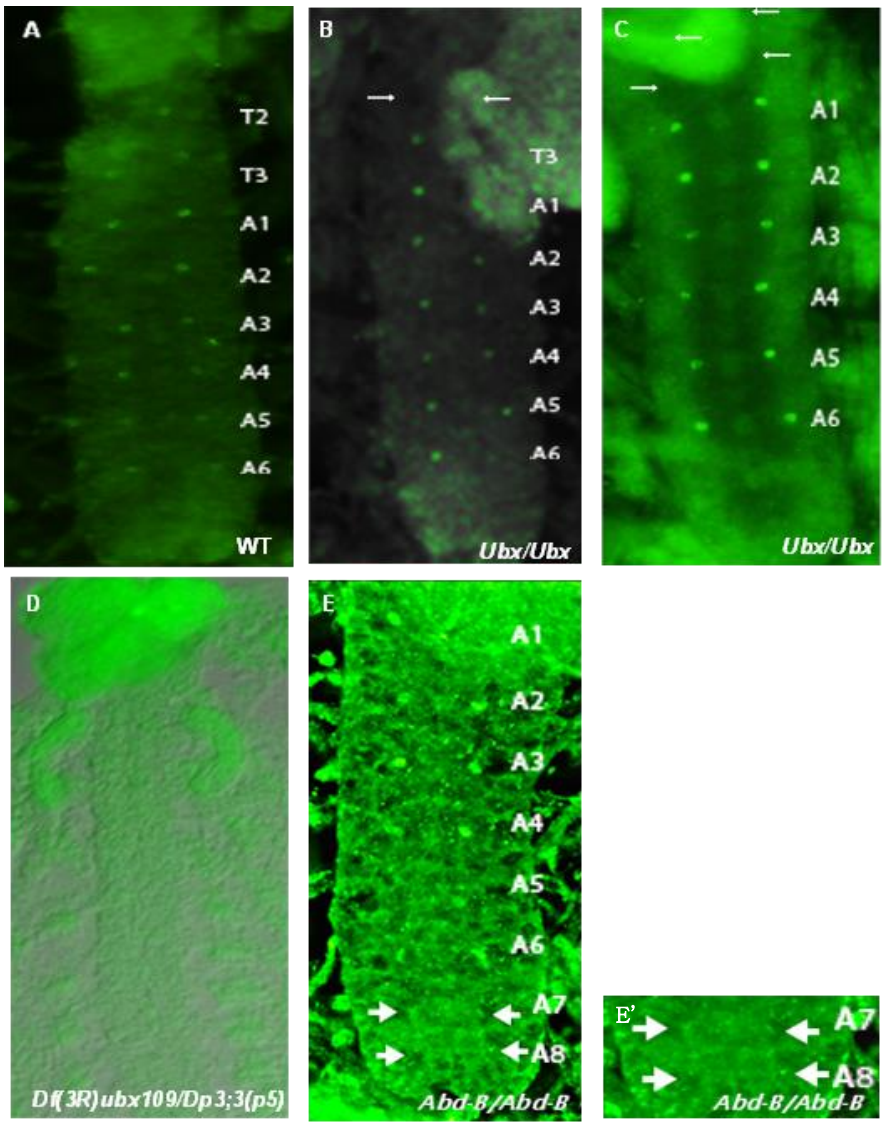


Figure 5. The expression of corazonin in null mutation of *Hox* genes

Since all strains of null mutant lines were embryonic lethal, the effect of loss-of-function only can be examined before hatch. The narrow white arrows indicate missing neurons. (A) Corazonin neurons were expressed in T2-A6 in wildtype. (B-C) The expression patterns of corazonin in *Ubx* null mutant. Corazonin neurons in brain lobes were intact but there were missing pairs in T2 and T3 in ventral nerve cord. (D) Only brain lobes remained intact and all corazonin neurons were missing in VNC. The image was overlayed with a DIC image to show the morphological position

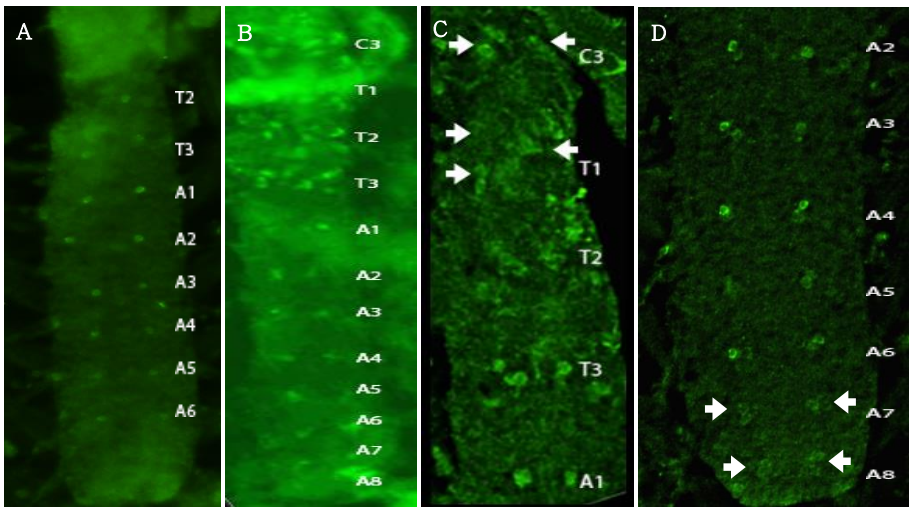
of expression domain. (E, high magnification in E') *Abd-B* loss-of-function mutant recovered the posterior corazonin cells in A7 and A8.

3.4. Occurrence of Programmed Cell Death in Early Embryogenesis

The cell death mechanism is well known in the post-embryonic stage. Some of vCrz neurons and the dorso-medial corazonin neurons are dead in early pupal development. If EW3 siblings are not dead by apoptosis, they differentiate to the corazonin producing neurons (Lee et al., 2012).

In embryogenesis, on the other hand, 30 neuroblasts (NBs) of T1-A8 segments in a stereotyped spatiotemporal pattern show normal generation of neural cells. Unlike the general NB map, the number of NB7-3 subsets is reduced in tail region (Technau et al., 2014). To figure out whether there are programmed cell death in embryogenesis, *p35* was driven by *eg-gal4* (Figure 6). Since *p35* forces to block all of the effect of caspase in apoptotic pathway, the overexpression of *p35* rescued the apoptotic cells in all segments.

As a result, the emergence of corazonergic neurons in *eg-GAL4/UAS*-p35 embryos is found not only in T2-A6, but also in C3-T1 and A7-A8 (Figures 6B-D). Especially the region of A7-A8 is overlapped with the posterior regional expression of *eg-GAL4/UAS-Abd-B*. Originally, some neuromers including NB7-3 are not generated in posterior end. It means programmed cell death occurs in the establishment of corazonergic neurons during early embryogenesis. Therefore, we concluded that *Abd-B* have a pro-apoptotic role in corazonin neural formation.



WT	*eg-GAL4 / UAS-P35*

Figure 6. Programmed cell death in corazonergic neuron development (A) The corazonin expression of wild type embryo at stage 16. (B) *eg-GAL4 / UAS-P35* embryo had extra coraznonin neuronal cells in C3-T1 and A7-A8. (C-D) White arrows indicate the newly detected corazonin neural cells which were survived by blocking apoptotic pathway. (C) High magnification in anterior part of *eg-GAL4 / UAS-P35* embryo. (D) High magnification in rear part of *eg-GAL4 / UAS-P35* embryo.

3.5. Manipulation of Corazonin Neuron Alters Time Dependent Ethanol-Induced Sedation

In recent study, males showed greater responses than females in both ethanol-induced hyperactivity and resistance to sedation. In other words, there was sex-specific dimorphism of corazonin-related behavioral response. Interestingly, when they were exposed to high dose of ethanol, their response was reversed. It meant when flies were exposed to 100% ethanol, males were more sensitive to ethanol-induced lethality than females (Devieni and Heberlein, 2012).

To examine sexually different behavioral response, control strains (Oregon-R, Canton-S, w^{1118}, WT crossed with *eagle*) were tested at first. As wild type's phenotype, three of four lines' male flies except Oregon-R were inclined to have a resistance to ethanol more than female. Thus, males appeared to be more sensitive to its sedative effect.

Compared to the wild type, on the other hand, the gain-of-function mutants which had alternated corazonin neurons show different sexually dimorphic responses in some extent (Figure 7). Both *eg-GAL4/UAS-Ubx* and *eg-GAL4/UAS-abd-A* were without distinction as to sex. That is, they had a tendency to have a resemblance to ethanol exposure. On the other hand, there were statistically significant differences in respect of sedation time/response among mutants and wild type (Figure 7D, $P < 0.0001$, two-way ANOVA). To figure out whether modified corazonin neurons affected on sedative response, the data were classified according to sex distinction. Interestingly, males were remarkably changed the degree of resistance to ethanol-induced sensitivity compare to that of wild type (Figure 8A), but females showed constant response relatively (Figure 8B).

Altered corazonin neurons of gain-of-function mutants in males were significantly different in the amount of sedative behavior measured by ST50, which dependent on their modified corazonin transcript level. ($P < 0.0001$, F = 40.66, R square =

0.9476, one-way ANOVA)

To be more specific, *eg-GAL4/UAS-p35* male showed fast loss-of-righting-reflex (LORR) and greatly decreased resistance to Ethanol exposure. This phenomenon was a bias against the previous research that male show more resistance to ethanol. In observation, when they were primarily exposed to vaporous ethanol, they showed increased hyperactivity compared to all other strains. In contrast, *eg*-GAL4/UAS-*Abd-B* which was represented to the reduced corazonin level was sedated faster than wild type. The corazonin expression of *eg-GAL4/UAS-Ubx* and *eg-GAL4/UAS-abd-A* mutants was increased so slightly that the meaning of ethanol induced sedation time would be negligible, compared to that of wild type.

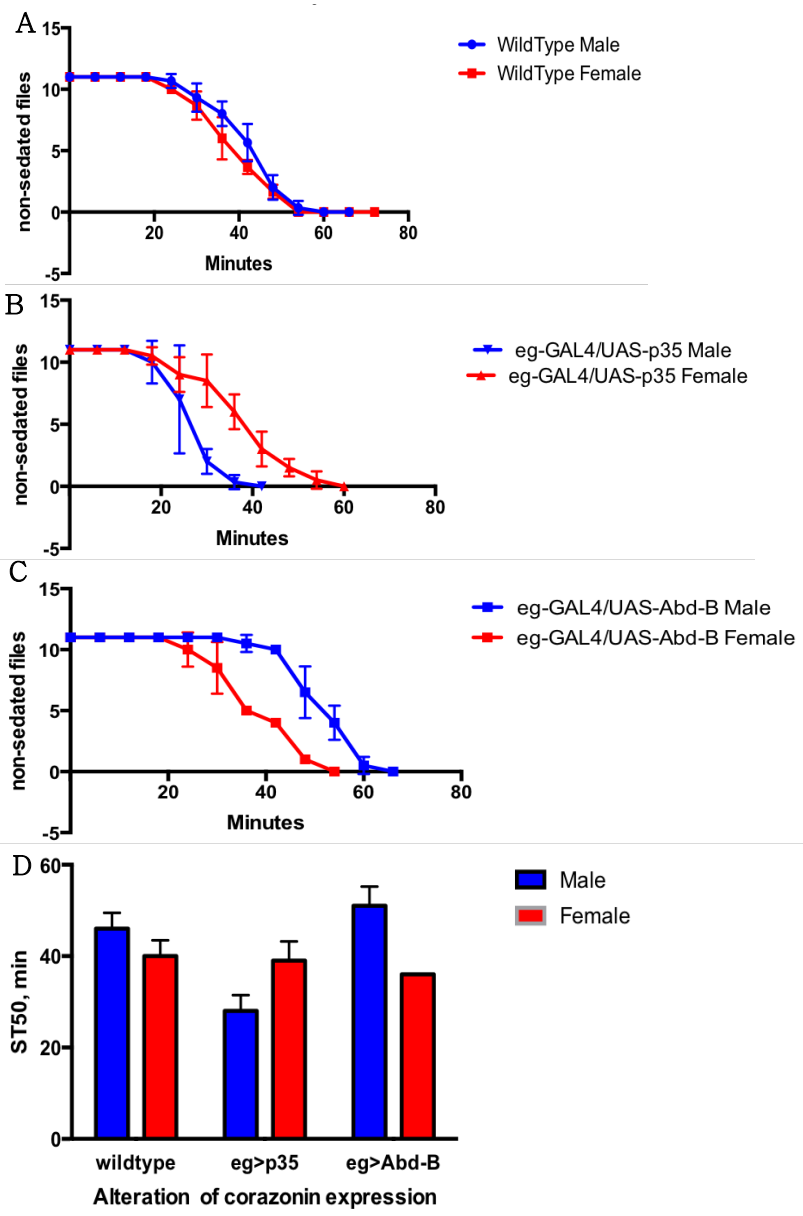


Figure 7. Ethanol-induced sedation in different sex (A-C) Sedation curves of males and females exposed to vaporized 85% ethanol. Males had greater ST50 than females except in *eg>p35 (eg-GAL4/UAS-p35).* (D) Alteration of corazonin neuron affected the ethanol induced sedation in mutant. After considering 2 factors, sex distinction and manipulation of corazonin neuron, the statistical analysis were performed ($P < 0.0001$, two-way ANOVA).

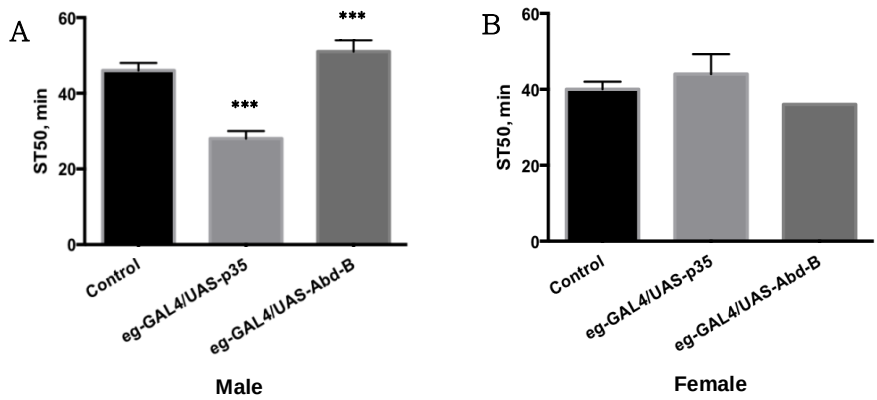


Figure 8. Male showed significantly different ethanol response in alteration of corazonin neurons. Male exhibited greater difference in ethanol induced sedation measured by ST50. (A) In males, the higher corazonin level caused hypersensitivity to ethanol exposure. (B) Females were inclined to maintain their response to ethanol regardless of changed corazonin expression. *** $P < 0.0001$, one-way ANOVA.

CHAPTER 4. DISCUSSION

The two main roles of *Hox* genes commonly known are determination of cell fate and causing programmed cell death in a segment-specific manner. In this study, three *Hox* genes, *Ultrabithorax (Ubx), Abdominal-A (abd-A) and Abdominal-B (Abd-B)* were chosen to understand how *Hox* gene acts on the identification of the corazonergic neuron in developmental stages.

Moreover, the major function of neuropeptide corazonin is regulating stress. Administering ethanol response was changed in various corazonin level caused by mutation.

4.1. *Hox* Genes and Specification of Neuroblast

Several studies recently mentioned *Hox* genes specify neuronal subtypes (Dasen, 2009; Rogulja-Ortmann et al., 2008). Specially, the motor neuron of NB7-3, generated from GW neuron, was produced in T1-A8 and promoted by *Antennapedia (Antp)* and *Ubx* (Rogulja-Ortmann et al., 2008).

Likewise there were similar events in developing corazonin neurons. Since the expression of corazonin in wild type was the same region of endogenous expression of *Ubx* and *abd-A*, the hypothesis that both *Ubx* and *abd-A* were candidates forming corazonin neurons comes to existence. Based on observation, the role of *abd-A* in corazonin neurons was hard to asses separately, but there were combinatorial interactions with *Ubx* in specialization corazonin neurons. In short, they participated in promoting corazonin neurons in specific thoracic and abdominal region, T2-A6.

The lack of *Ubx* deformed the T2-T3 corazonin neurons, which means *Ubx* specialized the T2-T3 segments strongly under the influence of *Abd-A*, mainly specifying the corazonin neuron in A1-A6. Moreover, ectopic expression of *Ubx* or *abd-A* caused the extension of anterior segment, T1. I gingerly suggest that *Ubx* and

abd-A promoted the corazonin neurons and also can trigger the transformation of NB7-3 sublineages. Considering all these factors, *Ubx* and *abd-A* may act on differentiating neural cells of NB7-3 into serotonin and corazonin neuron in late stage embryo. To find out the cell fate determinant in embryogenesis would be a further challenge (Figure 4).

4.2. *Hox* Genes and Programmed Cell Death of Corazonin Neurons in Embryogenesis

Programmed cell death (PCD) in the generation of segmental diversity was commonly observed. In NB7-3, it is possible that PCD causes removal of either corazonin neuronal precursor, GMC3, or the neuromers secondly divided from non-corazonergic neuromers.

As conclusion, the lack of corazonin neurons in C3-T1 and A7-A8 segments was caused by programmed cell death in early embryogenesis based on the result of ectopic expression of *p35.* For the absence of corazonin neurons in A7 and A8, *Abd-B* could be responsible for pro-apoptotic roles in these segments. The reason is that ectopic overexpression of *Abd-B* triggered cell death in anterior region. The second is that the loss of *Abd-B* function keeps corazonin neurons alive in the caudal abdominal segments. However, not all corazonin cells were missing in ectopically overexpression of *Abd-B* so there were also other interactions in existence of corazonin neuron in anterior segment specification.

Moreover, observation of 3 corazonin neural cells in each hemisegment of *eg-GAL4/UAS-p35* embryos suggested that the 2 other extra cells were not because of the cell death of corazonergic neural precursors. In previous research, *grim*, one of the *Drosophila* cell death genes plays an indispensable role in developmental PCD of corozonin neuronal precursor cells (Lee et al., 2013). When a universal inhibitor of caspase, *p35*, was expressed in entire NB7-3 lineage including both undifferentiated and differentiated progeny, the extra corazonin neurons were expressed only in normal

corazonergic neuromeres (Novotny et al., 2002; Lundell et al., 2003; Lee et al., 2013). Therefore, the absence of corazonin neurons from non-corazoneric neuromers was not due to its precursor in early embryogenesis.

In summary, the lack of corazonin neurons in C3-A1 was not likely due to *Abd-B* induced programmed cell death, while the absence of corazonin neurons in posterior segment was caused by pro-apoptic *Abd-B* in embryogenesis. Furthermore the death of the extra corazonin neural cells in each hemisegment was not related to their precursors (Figure 9).

4.3. Ethanol Response in Alteration of Corazonin Neuron

In *Drosophila*, similar to human, there were acute response to high doses of alcohol induce sedation, on the other hand, low doses increased locomotor activity (Devineni and Heberlein, 2012; McClure and Heberlein, 2013).

Also the corazonin related stress responses were sex-dimorphic (Zhao et al., 2010). In this study, ethanol induced sedation show sex-specific and male had more resistance to ethanol response than female in normal corazonin expression. However, male with increased corazonin expression was more sensitive to ethanol-induced lethality than female in administering high concentration ethanol. I suggested that this counter-productive behavior in ethanol exposure appeared on male remarkably.

In recent research, the absence of corazonin neuron by cell death *(crz-GAL4/UAS-rpr)* extended lifespan (Zhao et al., 2010) and the loss of corazonin, as well as corazonin receptor in DLPs (dorso-lateral peptides) resulted in the increased stress resistance and metabolic component levels. Also corazonin is probably involved in the physiological regulation of the heart beat as well as stress resistant response by regulating corazonin transcription in a cell specific manner (Choi et al., 2005). Based on the previous

description, increasing corazonin level can affect cardio-acceleration and their lifespan. One reason is that an organism which has the increased level of corazonin moves so vigorously that consume more energy, and depletion of oxygen may lead them to faster desiccation.

Secondly, in female, corazonin expression or its secretion can be normally inhibited to impede hyper-sensitivity under the stressful condition like administration of high concentration ethanol. Expression of Tra (*transformer*) in developing nervous system contributed to the sex difference in ethanol sedation, whereas the sex difference in ethanol hyperactivity may arise from Tra function outside the Nervous system (Mclure and Heberlein, 2013). Likewise, female specific gene can contribute to mimic the normal action of ethanol induced stress and sustain stable condition.

In short, the continuing high concentration alcohol exposure to male caused more severe response than female, depending on the fluctuation of corazonin level by artificial manipulation.

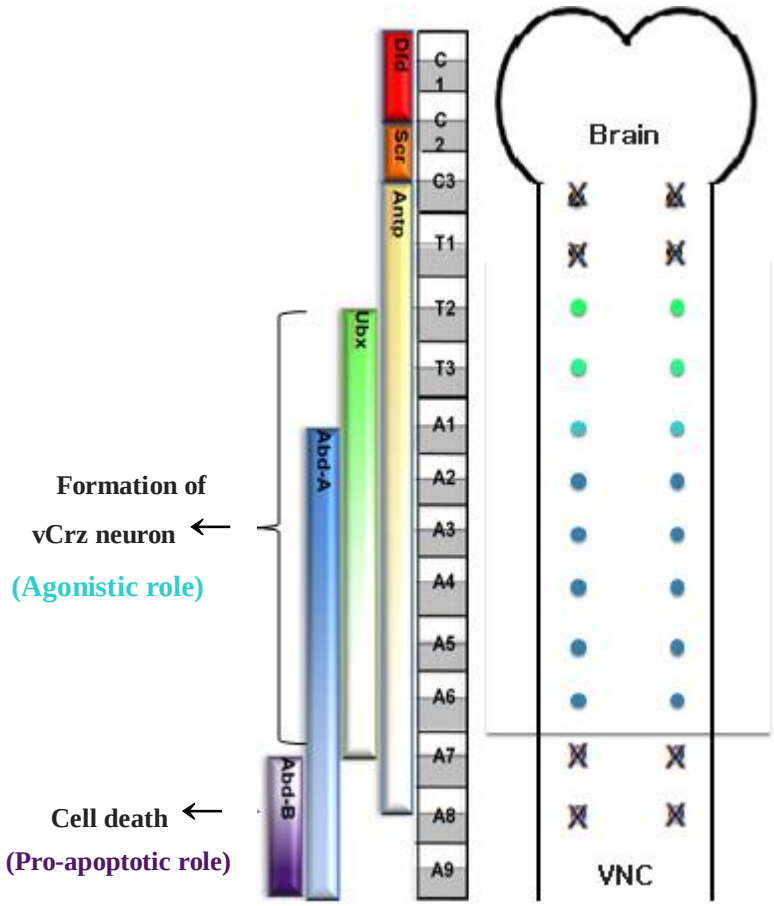


Figure 9. The spatiotemporal pattern of embryonic corazonin expression The diagram expressed the corazonergic neuron formation in VNC during embryogenesis. Both *Ubx* and *abd-A* had a positive effect on corazonin neural formation, while *Abd-B* exerted a pro-apoptotic role.

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국문초록

신경아세포 7-3 계보인 Corazonin 신경에서 Hox 유전자의 역할

초파리의 발생과정에서 hox 유전자는 체절 특이적인 세포의 운명을 결정하는데, 최근 신경세포의 세포사멸 기작에 Hox 유전자가 관여하는 것으로 밝혀졌다. 본 연구를 통해 신경아세포 7-3 계보인 코라조닌 신경세포에서 Hox 유전자에 의한 체절특이적인 세포 운명 결정과 사멸이 배아발생시기에서 일어남을 알 수 있었다. *Ubx* 와 *Abd-A* 두 유전자가 코라조닌 신경이 T2-A6 체절에 특이적으로 발현하도록 촉진하는 역할을 하는 것으로 나타났다. 한편, 배아 후측편에 해당하는 부체절 10-14 에 특이적으로 발현하는 *Abd-B* 의 과발현은 앞쪽 체절의 부재를 야기했다. 또, *Abd-B* 의 기능이 상실 되었을 때, 기존에 나타나지 않던 A7-A8 체절에 코라조닌 신경이 나타났다. 이는 세포자살을 억제하였을 때 회복된 부분과 일치하며, *Abd-B* 가 코라조닌 신경 형성에 있어 세포사멸인자로서 작용한다고 볼 수 있다. 더불어 스트레스를 조절한다고 알려진 코라조닌 신경이 Hox 유전자의 돌연변이로 바뀌었을 때, 초파리 성체에 어떤 영향을 미치는지 알아보았다. 코라조닌 발현이 증가함에 따라 알코올에 대한 민감성은 커졌고 성별에 따라 상이한 반응을 보였다. 수컷이 암컷보다 알코올에 저항성이 더 큰 경향이 있으며, hox 유전자 돌연변이에 의한 코라조닌 발현 정도에 따라 유의미한 차이를 보였다.

주요어: 신경아세포 7-3, 코라조닌, Hox 유전자, 에탄올 반응, *Drosophila melanogastor*

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