CHAPTER I
INTRODUCTION

1.1 Reproductive biology of *Penaeus monodon*

Black tiger shrimp (*Penaeus monodon*) is a heterosexual aquatic animal. Commonly, the size of female is larger than male, with female reaching up 33 cm in body length. Females have receptacle (thelycum) with lateral plates that enclose the spermatophore. Mating occurs in their intermolt period during which female's cuticle is still soft. Female's reproductive organs consist of paired ovaries and oviducts. The ovaries are partly fused in the cephalothoracic region and composted of several lateral lobes that are bilaterally symmetrical extending from the pericardial region of the stomach to the telson (1). Ovarian maturation is classified into five stages based on size and histology. The first stage (undeveloped stage); the ovary looks like a thin opaque line which is visible along the length of the shrimp. The primary oocytes are larger than oogonia but smaller than oocytes in the perinucleolar stage, containing single to several nucleoli in nucleoplasm. The primary oocyte is surrounded with a layer of follicle cells which are either rectangular or cuboidal in shape. However, although developing eggs of this stage are increasing in size but they are not as yet producing yolk, and are known as previtellogenic oocytes. The second stage (developing stage); the ovary is thicker than stage 1 and can be visualized as a large centrally located opaque rope-like structure. The oocytes are developed and migrated out of margin of the ovarian lobes. The follicle cells are attached to periphery of each oocyte during migration. The follicle cells produce the yolk that is accumulated in the oocytes, during the process called vitellogenesis. The ovary in stage 2 is visualized through the dorsal exoskeleton as a thick, solid and dark linear band and expands at the posterior thoracic and anterior abdominal regions. Deposition of carotenoid pigment mainly causes the dark green color of the ovary in stage 3 or nearly ripe stage. By the end of vitellogenesis, the eggs develop cortical granules filled with jelly-like substance to form part of the egg shell membrane after ovulation. Subsequently, the

ovary becomes larger, prominently thick and appears a dark green colour and expands at the first abdominal segment with an enlarged bulbous region directly behind the carapace, this ovarian stage is pre-spawning stage or ripe stage (stage 4). The last ovarian stage is spent stage; after the release of the fully mature oocytes into to the oviduct at ovulation or complete spawning, the ovary at this stage looks similar to the ovary in undeveloped stage (2-4).

1.2 Neurohormonal regulation of female crustacean's reproduction

Reproduction in crustacean has been hypothesized to be mainly controlled by dual antagonistic hormones; inhibitory and stimulatory hormones. Gonad inhibiting hormone or GIH (also known as vitellogenesis inhibiting hormone, VIH) is released from the X-organ-sinus gland complex located in the optic ganglia of the eyestalks. The function of GIH is inhibition of ovarian maturation in vitellogenesis process. GIH exerts its action through target tissues such as hepatopancreas and ovary which are sites of yolk protein synthesis (5). Because the eyestalk is the site of GIH synthesis, eyestalk ablation is commonly used to induce effective ovarian maturation in many species of penaeid shrimp in captivity as it reduces the synthesis of GIH and thus stimulates vitellogenesis. In addition, there is another hormone in crustacean central nervous system which is supposed to act as gonad stimulatory hormone (GSH). This putative GSH is assumed to be synthesized in the brain and thoracic ganglia which may directly contribute to stimulation of ovarian maturation (6). This process may involve several biogenic amines which mainly function as neuroregulators including dopamine (DA), serotonin (5-HT), octopamine, histamine, noradrealine (norepinephrine), adrenaline (epinephrine), tryptamine and tyramine. However, several studies hypothesized that 5-HT and DA are the two biogenic amines that are able to modulate reproduction processes in crustaceans; 5-HT generally stimulates ovarian maturation whereas DA plays the opposite role (7).

1.3 The role of dopamine neurotransmitter

Dopamine (3,4-dihydroxyphenethylamine, DA) is a biogenic amine neurotransmitter that is synthesized from tyrosine in two steps reaction; tyrosine is first catalyzed by tyrosine hydroxylase into L-DOPA which is further decarboxylated by DOPA decarboxylase to DA (8) as shown in figure 1.1. DA plays an important role in several brain functions to regulate a variety of physiological and behavioral activities in both vertebrates and invertebrates. In mammals, DA plays various functions including locomotion, cognition, emotion, positive reinforcement, food intake and endocrine regulation (9). In the nematode *Caenorhabditis elegans*, DA is involved in male mating behavior, locomotion and egg laying (10). DA is also detected in many insect species, in which its mechanism is operated through the brain. In the honey bee, *Apis mellifera*, DA is associated with olfactory learning and memory (11). In *Drosophila*, DA has important role in larval molts, pupariation and adult emergence in development (12). Furthermore, DA is widely distributed in the central nervous system of decapod crustaceans and acts as neurotransmitter/ neuromodulators and as a hemoymph-borne neurohormone (13-14). DA has been reported to stimulate the release of both the pigment-concentrating hormone (15-16) and the distal retinal pigment dark-adapting hormone in the fiddler crab, *Uca pugilator* (16). It also caused the release of crustacean hyperglycemic hormone from the X- organ-sinus gland complex of *Orconectes limosus*, the shore crab, *Carcinus maenas* (14), the black tiger shrimp, *P.monodon* (17), and freshwater giant prawn, *Macrobrachium rosenbergii* (18). In addition, DA is involved in modulation of the physiological response and suppression of the immune ability such as increased susceptibility of the whiteleg shrimp to *Vibrio alginolyticus* (19-20) and *M. rosenbergii* to *Lactococcus garvieae* (21) as well as increases mortality in *P. monodon* which is challenged with *Photobacterium damsel* pathogen (22). Furthermore, DA also plays an important role in crustacean reproduction. It is hypothesized that DA either stimulates the release of GIH from the eyestalk neuroendocrine system or inhibits the release of GSH or both. DA has so far been found to antagonize the gonad-stimulation action of 5-HT, for example to inhibit 5-HT stimulated testicular maturation in *Procambarus clarkii* (23) and ovarian maturation in *U. pugilator* (24), *P. clarkii* (25) and *M. rosenbergii* (26).

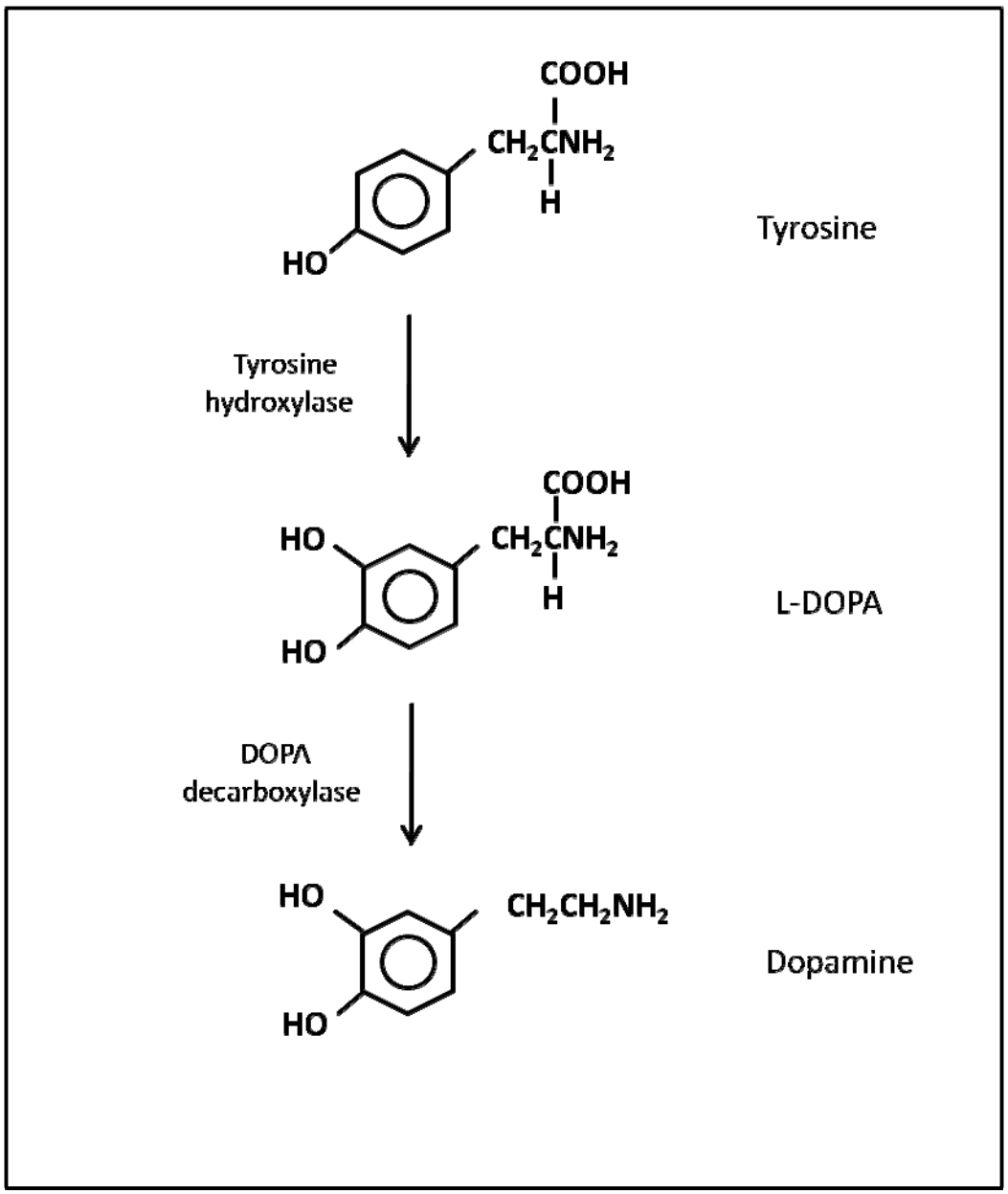

Figure 1.1 Biosynthesis of dopamine (DA). Tyrosine is hydroxylated by tyrosine hydroxylase to 3, 4-dihydroxy-L-phenylalanine (L-DOPA). Decarboxylation of L-DOPA by DOPA decarboxylase finally generates dopamine.

1.4 Characteristic of G-protein coupled dopamine receptor

Dopamine receptors (DARs) are members of rhodopsin-like family of seven transmembrane (TM) domains G protein-coupled receptors (GPCRs). The transmembrane domains (TM1-TM7) are connected by three intracellular loops (i1, i2, i3) and three extracellular loops (e1, e2, e3). The DAR is stabilized by a disulfide bond formed between cystein residues in i2 and i3 (27). The structure of DARs comprises of ligand-binding site, an extracellular amino-terminus and an intracellular carboxyl-tail that often contains consensus sequence motif for N-linked glycosylation (28). DARs share relatively high homology within their TM and share several conserved residues which are required for binding to catecholamine, a group of neurotransmitters that contain 3, 4 hydroxyphenyl group including DA. In particular, two serine residues in TM5 are involved in recognition of the two hydroxyl groups of catecholamines. An aspartic acid residue in TM3 is most probably involved in binding to the amine group of the cathcholamine side chain and a phenylalanine residue in TM6 that interacts with catcholamine aromatic ring contributes stabilizing orthogonal interaction with the aromatic moiety of ligands. An aspartate residue in TM3 and the DRY sequence following TM3 are involved in receptor activation (29-30). These receptors contain several phosphorylation sites in the i3 and C-terminal tail regions, which are believed to be important for phosphorylation induction in G-protein coupling, desensitization and endocytosis (9). The binding of ligand to DAR occurred in the pocket formed by the TM region in the plane of the membrane. The specific residues in each of these TM domains provide specific properties to ligand binding in receptor activation, which are mediated by intracellular signaling pathway as described below.

1.5 Signaling pathway of GPCRs

GPCRs exist as different receptor families characterized by sequence similarity, length and function of their N-terminal extracellular domain, C-terminal tail and intracellular loops. Each of these domains provides specific properties to various receptors. The GPCRs are involved in the recognition and transduction of various messengers such as light, Ca^{2+}, odorants, amino acid residues, nucleotides, peptides

and proteins (28). These messages control diverse functions in the cells; for example enzyme activity, ion channels and transportation of vesicles through the catalysis of the GDP-GTP exchange on the heterotrimeric G-protein (Gα-βγ). Heterotrimeric GTP-binding proteins (G-proteins) are critical component of signal transduction pathways that receive and carry information from various factors to the appropriate cellular response. G protein is composed of an α-subunit (including two domains, a GTPase domain that is involved in the binding and catalysis of GTP and a helical domain that buries the GTP within the core of the protein) and βγ subunit forming an undissociable complex (28-30). Diverse functions of the G protein in mediating signaling system define the specificity of each receptor in intracellular signaling regulation. There are numerous subtypes of G proteins; the α-subunits can be divided into four families including $G\alpha_s$, $G\alpha_i/G\alpha_o$, $G\alpha_q/G\alpha_{11}$ and $G\alpha_{12}/G\alpha_{13}$. Each family contains several members based on structure similarity and often shares some of their functional properties, which may play a role directing specificity of the receptor and effectors. The βγ-complex of mammalian G protein also contains several members; there are five β-subunits and twelve γ-subunits (31). Activation of the receptor occurs when a ligand interacts with heptahelical receptor on the cell surface. The ligand either stabilizes or induces a receptor conformation and lead to activation of G proteins on the inner membrane surface of the cell, which then catalyze the exchange of GDP to GTP on the Gα subunit resulting in the dissociation of Gα from Gβγ as well as the receptor activation. The GTP bound Gα subunit can regulate several intracellular effectors such as adenylyl cyclase, phospholipase Cβ, K^+ and Ca^{2+} channels, and cyclic GMP phosphodiesterase. The Gβγ also plays active roles in signal transduction such as regulation of K^+ channels and phospholipase Cβ (32). However, the system is inactive when the GTP is hydrolyzed by GTPase activity to GDP and leads to reassembly of Gα and Gβγ complex until the system returns to its resting state. Figure 1.2 shows signaling pathway of GPCRs to intracellular response.

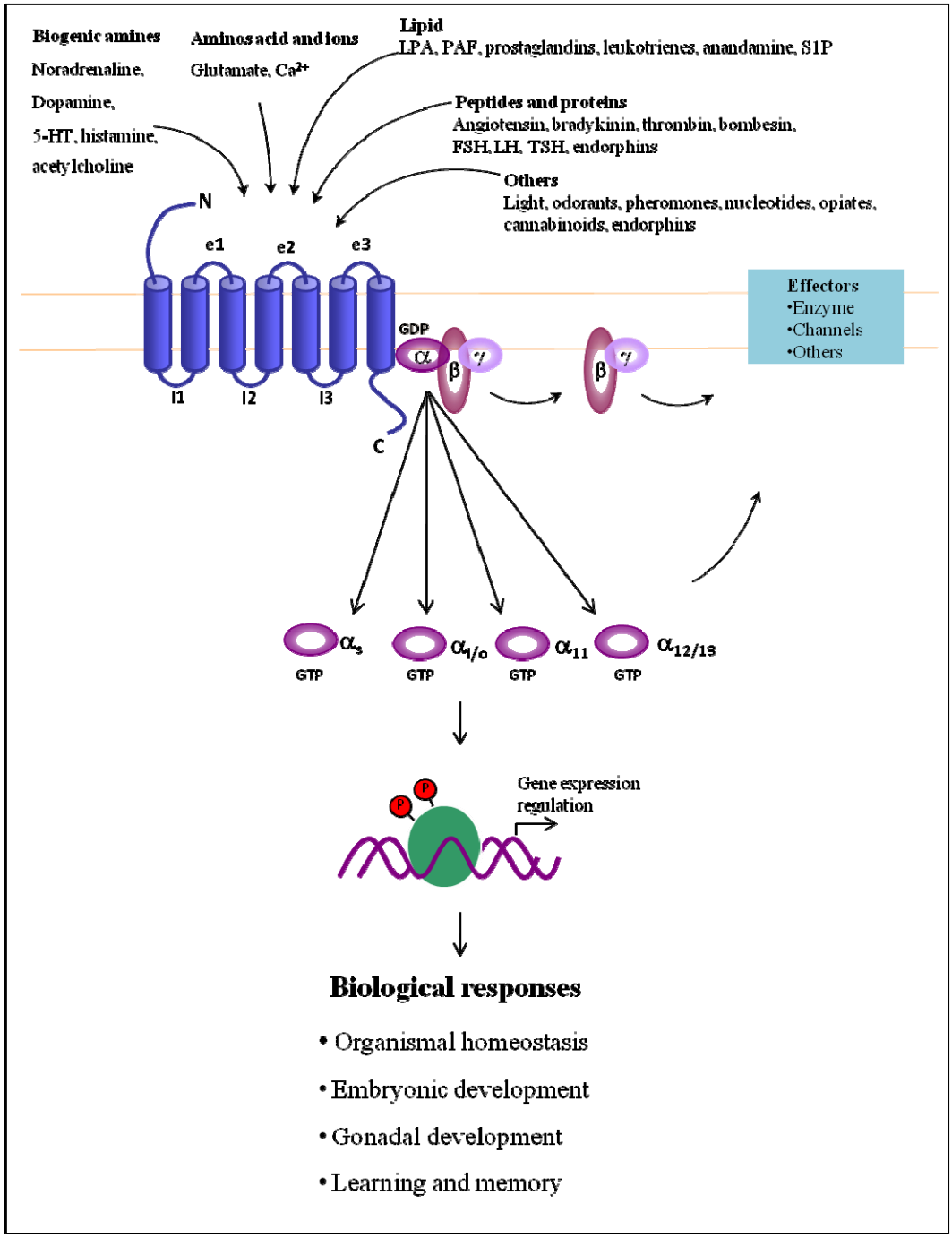

Figure 1.2 The signaling pathway of G protein coupled receptors (GPCRs). GPCR is connected with three extracellular loop (e1, e2 and e3) and intracellular loop (i1, i2 and i3). The GPCR structure contains an extracellular amino-terminus, an intracellular carboxyl-tail and ligand-binding sites. The receptors are activated with various factors and mediated intracellular signaling.

1.6 Classification of DARs

DARs in vertebrates and invertebrates are generally classified into two groups based on sequence similarity, functional characteristics and pharmacological properties; D1-like and D2-like receptors (9). Although the vertebrate and invertebrate DARs are classified in parallel hypothesis but the orthologous relationships in vertebrate and invertebrate DARs have been still unknown. Some studies suggested that pharmacological profile of the vertebrates DAR was not conserved across those of invertebrates for example, vertebrate pharmacology could not be used to classified arthropod DARs (33). Recently, DARs can be defined into subtypes within each group. In vertebrates, DARs contain two subtypes of D1-like receptor and three subtype of D2-like receptor whereas the invertebrate DARs comprise two subtypes of D1-like receptor and one subtype of D2-like receptor (34).

1.6.1 DARs in vertebrates

The vertebrate DARs can be divided into two types; D1-like receptor and D2-like receptor. The D1- and D2-like receptors clearly differ in sequences and structures. The D1-like receptors contained a short i3 loop and its C-terminal tail is very long whereas, the D2-like receptors exhibit a longer i3 loop but a short C-terminal end. Most differences occurred in the i3 loop and the C-terminal tail, which are widely believed to be involved in coupling with heterotrimeric G-protein of each receptor (35). The gene encoding D1-like receptors does not contain intron whereas D2-like receptor gene contains several large introns (9). In addition, DARs are also defined by their ability to regulate adenyl cyclase activity and cAMP accumulation in the cell. The D1-like receptors interact with the $G\alpha_s/G\alpha_{olf}$, activating adenylyl cyclase and leading to an increase in intracellular cAMP levels whereas the $G\alpha_i/G\alpha_o$ coupled D2-like receptors inhibit adenylyl cyclase and decrease cAMP levels (36-37). In addition, the pharmacology is also contributed to classification of the differences of DARS. The D1- and D2-like receptors contained distinct structural characteristics and functional properties that require different ligands (38). Recently, the vertebrate DARs are finely divided into two subtypes (D_1 and D_5) of D1-like receptor and three subtypes (D_2, D_3 and D_5) of D2-like receptor. The detail of each subtype has not been well defined, but only a little difference is reported between groups (9).

1.6.2 DARs in invertebrates

Similar to vertebrate DARs, D1-like receptor in invertebrate can be classified into two groups. The first group is DOP1 group; most closely related to the vertebrate D1-like receptors in the modulation of an increase of intracellular cAMP. Members of these groups include AmDOP1 of honey bee *Apis mellifera* (39-40), CeDOP1 of *C. elegans* (41-42) and DmDOP1, also known as dDA1 of *Drosophilla* (43-44), $D_{1\beta\_Pan}$ of the spiny lobster *Panulilus interruptus* (33) and BmDopR1 of the silk worm *Bombyx mori* (45). The second group is invertebrate DARs (INDRs) such as AmDOP2 from honey bee and DAMB, also known as DopR99B (46-47) from *Drosophila*, $D_{1\alpha\_Pan}$ of *P. interruptus* (33) and BmDopR2 of *B. mori* (45). This group is closely related to the invertebrate octopamine receptors more than members of the DOP1 group and the vertebrates D1-like receptors. However, although the structure of INDRs differ from DOP1 group, they up-regulate cAMP levels as the D1-like receptor.

The invertebrate D2-like receptors contain members from *C. elegans*; CeDOP2 and a putative DAR CeDOP3 (48-49) the fruit fly; DD2R receptors (50) the honey bee; AmDOP3 (51) and $D_{2\alpha\_Pan}$ of *P. interruptus*. (52) The members of this group share high homology and activate the down regulation of cAMP levels like vertebrate D2- like receptors.

1.6.3 Functional properties and signaling transduction of DARs

Signaling transduction pathway is one of the criteria in DARs classification. According to the classification of DARs, they are classified into two classes (D1- and D2-like receptors) as described above. DARs are member of GPCRs family, their signaling are primarily mediated by interaction and activation of heterotrimeric G protein. The D1-like receptors couple to $G\alpha_s/G\alpha_{olf}$ and lead to an increase in cAMP levels and protein kinase A (PKA) activity, alternatively the D2-like receptors couple to $G\alpha_i/G\alpha_o$ protein to decrease cAMP levels and PKA activity (9, 32). The signaling modulation of DARs has been demonstrated by several studies. For examples, DARs have been shown to couple with multiple G proteins in various heterologous systems and then mediate subsequent signaling in response to ligands. Both the Gα and Gβγ subunits are mediated by individual responses and the activated

G protein subunits can directly interact with target proteins such as ion channels without altering second messenger levels. Furthermore, GPCRs can activate other pathway, like the mitogen activation protein kinase (MAPK) pathway via crosstalk and also directly activate G protein (53-55). The functional properties of DARs are mediated by several signal transduction pathways as summarized in table 1.1.

Table 1.1 Classification and general functions of DARs (9, 32, 38)

Class	General functions
Vertebrate D1-like receptors - D1 - D5 Invertebrate D1-like receptors - DOP1 - INDR	1.Stimulation of adenelyl cyclase and increase cAMP levels 2.Stimulation of phopholipase C mediated mobilization of intracellular Ca^{2+} 3.Modulation the activity of Ca^{2+}, K^+ and Na^+ channels 4.Inhibition of the activity of the Na^+/H^+ exchanger by both cAMP dependent and cAMP independent mechanism 5.Regulation of other receptor such as glutamate receptor and GABA receptor 6.Regulation of other signaling pathways - Inhibition of Na^+- K^+- ATPase -Activation of Mitogen-Activated Protein Kinases (MAPK)
Vertebrate D2-like receptors - D_2 - D_3 - D_4 Invertebrate D2-like receptor - D2-like	1.Inhibition of adenelyl cyclase and decrease cAMP level 2.Modulation of voltage-gated ionic channels - Inhibition of Ca^{2+} ionic channels through $G\alpha_o$ - Activation of K^+ through $G\alpha_i$ 3.Other functions such as mobilization of intracellular Ca^{2+} store, PKC activation, release of arachidonic acid, Na^+/H^+ exchange, inhibition of Na^+- K^+- ATPase

1.7 Distribution and localization of DARs

DARs distribution is studied in many vertebrates and invertebrates. The D1- and D2-like receptors are mainly distributed in the central nervous system, especially in the brain region. In human, D_1 receptor is the most highly expressed DAR rather than other subtypes. Co-expression between D_1 and D_2 subtypes is detected in the same region of the brain. In insects, DARs are mainly located on the mushroom bodies in brain. Presumably, the mushroom bodies are regions of the brain where biogenic amine acts through regulation of cAMP and may play important roles in learning and memory and is also implicated in the regulation of complex behaviors (56). The expression of DARs in insects is exhibited in various species. The honeybee Am*dop1* mRNA is expressed in the mushroom bodies and in other brain regions including the antennal and optic lobes, throughout metamorphic development, while *Amdop2* is widely expressed in the brain and is involved in brain development (39, 57). In *Drosophilla* embryos, the *Dmdop*1 transcript is present in early syncitial stages, cellular blastoderm stages and extending germ band, suggesting that it may play a role in early development (44). Besides, expression of DARs in *C. elegans* (*CeDop1* and *CeDop3*) also showed overlapping localization in the same cell (48-49). In decapod crustaceans, little is known about the localization of DAR. However, most studies reported that DA is mainly distributed in the central nervous system, brain and ovary and is widely hypothesized to play roles in the reproductive process. For example, DA stimulates testicular maturation in *P. Clakii* (23) and is involved in vitellogenin synthesis of ovarian maturation in *U. pugilator* (24) and *P. clarkii* (25). In *M. rosenbergii*, DA may be involved in ovarian maturation by acting through DAR type 1 (26). Therefore, the expression of DA may also suggest the tissue localization of DARs in crustacean.