Metazoan invertebrate and vertebrate nervous systems evolved mainly to control movements to help animal forage and escape from predators. Describing how various neural pathways interact and converge their output onto motor neuron, to generate output, Sir Charles Sherrington said, ‘Before finally converging upon the motor neurone the arcs converge to some degree. Their private paths embouch upon internuncial paths common in various degree to groups of private paths. The terminal path may, to distinguish it from internuncial common paths, be called the final common path. The motor-nerve to a muscle is a collection of final common paths’ (Sherrington, 1947).

Less evolved brains exhibit minimal complexity and more stereotypic behaviours. Sherrington observed as the animal is higher up in the evolutionary tree, the voluntary control of brain over rhythmic movements increases progressively: ‘Reflex action presents certain advantages for physiological description. It can be studied free from complication with the psyche: also free from complication by that type of ‘nerve’ activity which is called autochthonous (or ‘spontaneous’) and generates intrinsically arising rhythmic movements, e.g. breathing, etc… Studied in that self-contained animal group, the vertebrates, behaviour seems to become less and less reflex as the animal individual becomes more and more complexly individuated. The spinal man is more crippled than is the spinal frog’ (Sherrington, 1947).

Compared to vertebrates, invertebrates with their less evolved brains, exhibit highly stereotyped behaviours. Invertebrate nervous systems are of ganglion type with the brain and ventral nerve cord that are the equivalents of the vertebrate brain and spinal cord. Within the central nervous system, stereotyped output generating circuits are localised to the ventral nerve cord region. A recent study demonstrated that the brain activity is dispensable for regular crawling movements in Drosophila larva (Berni et al., 2012). This diminished control of brain over ventral nerve cord can be explained by sensory perception dependent mechanisms. Sensory stimuli forms two kinds of representations in the animal nervous system: organized sensations (vision and smell) and amorphous sensations (thermal and tactile) (Cajal et al., 1999). The brain
mainly receives vision and smell-related sensations, creates a perfectly organised image of the external world with clarity and space-time relations which have the ability to form associations with the objects in their environment. The associations formed by the neurons in brain and the output of their higher dynamic interactions, inhibitory or excitatory, may direct the animal away or towards the objects in its niche. In this manner insect larval brain chiefly function to provide directionality during locomotion without interfering with regular exploratory functions related to the locomotion pattern generator. The ventral nerve cord mainly receives tactile and thermal sensations, which are diffuse, with no detailed relation of size and shape. *Drosophila* ventral nerve cord localised neural circuits are less influenced by brain-derived signals and are ideal for studying interactions of neurons.

A small number of neurons, 10,000 in larval ventral nerve cord (Ohyama et al., 2013), and minimal brain influence on movement generation makes *Drosophila* larval nervous system an ideal model for studying neuronal connectivity of locomotion.

1.1 The main objectives of this thesis are:

1) To test the *Drosophila* Vesicular glutamate transporter (*DVGlut*) gene regulatory region driven expression in subsets of motor and interneurons

2) To study the organization of glutamatergic larval motor and interneurons in relation with sensory neuron representations and

3) To identify the function associated with the glutamatergic class of interneurons.

Regulation of insect gene transcription displays modular nature, with cis-regulatory regions spread or present between coding regions (Davidson, 2001; Wilson et al., 1990). All glutamatergic neurons contain Vesicular glutamate transporter (Vglut) which is essential to fill the synaptic vesicles with glutamate (Daniels et al., 2006). The fact VGlut is localised to all *Drosophila* motor neurons and six embryonic interneurons (Mahr and Aberle, 2006), suggests the probable presence of regulatory elements to control *DVGlut* expression in these neurons. Taking the advantage of gene regulation mechanism where an identified DNA sequence can be tested for enhancer function, enhancer-reporters from *DVGlut* gene regulatory region were generated.
Analysis of the expression patterns identified DVGlut enhancer-reporters label inter and motor neurons. Their anatomy suggested the presence of class specific domain organisation for motor neurons and putative glutamatergic interneurons. Localization of neurite patterns suggested the interaction of type II motor neurons with the dbd sensory neurons.

Positioned anatomically at the interface between sensory and motor neurons, interneurons play a key role in organizing behaviours. Detailed behaviour associated with a class of glutamatergic larval interneurons are studied in detail. Based on our observations along with the current literature a model was proposed to explain the observed behaviour.

One of the main functions of the nervous system is communication. To elicit a particular behaviour, neurons in the nervous system not only communicate with each other but also with other cell types like glia and muscle. In the following section, the life cycle of Drosophila melanogaster and the elements of the nervous system involved in locomotion are described.

1.2 The life cycle of Drosophila melanogaster

The genus Drosophila belongs to the phylum Arthropoda, members of which display segmented body and jointed appendages.

Figure- 1.1.
Figure- 1.1. The life cycle of *Drosophila melanogaster*. *Drosophila* is a holometabolous insect with distinct larval, pupal and adult life forms; completes life cycle in 10 days. Adult *Drosophila* shows sexual dimorphism with separate male and female forms.

The species *melanogaster* group is considered originated in South-East Asia, displays sexual dimorphism in abdominal colouration with male abdomen having entirely shiny black apical segments and female abdominal tergites with only dark posterior bands (Ashburner et al., 1986)(Figure 1.1). At room temperature (25°C), its generation time is about ten days with three distinct life forms – larva, immobile pupa and walking adult (Figure 1.1). Members of the genus *Drosophila* are commonly called fruit flies referring to the typical feature of many species found around ripe or rotten fruit.

1.3- *Drosophila* larval neuromuscular system

*Drosophila* larva is a bilaterally symmetrical animal; broadly divided into head, thorax and abdomen. The thorax has three segments (T1-T3) and abdomen nine (A1-A9), along the anterior-posterior axis. *Drosophila* larval nervous system controlling locomotion divided into the central and the peripheral nervous system. The central nervous system (CNS) consists of the brain and ventral nerve cord (VNC) (Figure 1.4). Both VNC and the body wall exhibit segmentation. In insect literature, one-half of a metamere or a segment of ventral nerve cord is called a neuromere. The outer layer of a neuromere occupied by cell bodies is cortex and inner region with axons is neuropile. The neuropile is a region where information processing occurs in the nerve cord. Functionally, *Drosophila* larval nervous system is organized to regulate movements and divided into three modules: the sensory, the motor and the interneurons. The motor neurons from a segmental region in the VNC innervate corresponding segment in the body wall (Figure 1.2 and 1.4). The VNC showing segmentation corresponding to body wall segments is depicted in Figure 1.2. A sensory neuron labelled green originates from the A2 segment of the body wall sends axonal projections to the A2 segment of VNC. A motor neuron localised in A4, labelled pink- has cell body in the A4 region of VNC and neuromuscular junctions in the A4-segment of the body wall (Figure 1.2). The body wall muscles along with VNC localized sensory, motor and interneurons of a hemi-segment forms a functional unit for locomotion (Niven et al., 2008).
Figure- 1.2. Schematic showing the larval central nervous system of *Drosophila.* *Drosophila* central nervous system consists of the brain and ventral nerve cord (VNC). The VNC harbours cell bodies of motor and interneurons and the axon terminals of sensory neurons. Motor neuron, labelled pink, has a cell body in the A4 segment of VNC, innervates A4 segment of the body wall. Sensory neuron, labelled green, has a cell body in the A2 segment of the body wall,
innervates A2 segment of VNC. Interneuron whose cell body labelled blue has arbours confined to VNC.

A hemi-segment of the larval abdomen has 30 body wall muscle (Landgraf et al., 1997; Sink and Whitington, 1991), 42 sensory neurons (Merritt and Whitington, 1995), 36 motor neurons (Hoang and Chiba, 2001), 32 glial cells (Ito et al., 1995) and 270-300 interneurons (Rickert et al., 2011).

1.3 a- *Drosophila* larval body wall sensory neurons

*Drosophila* larva perceives body wall sensory information by neurons located in the sense organs. Based on the sensory modality, two main types of sense organs are observed: 1) external sense organs (es) and 2) chordotonal- stretch receptor (ch) organs. The external sense organs are located in body wall hair function as mechanoreceptors to detect tactile information while the chordotonal- stretch receptor organs function as proprioceptors to detect the internal state.

**Figure- 1.3.**

*Drosophila* motor neuron dendrites, labelled blue, occupy the dorsal region in VNC and sensory neurons, labelled grey, ventral region. The sensory neurons belonging to a particular modality...
(proprioception, mechanosensation and nociception) occupy distinct regions (labelled dbd, ch and Class IV md respectively) in VNC.

The dbd sensory neurons enter dorsally while ch and md neurons enter CNS ventrally into neuropile (Figure 1.3). Based on the number of dendrites a neuron possess sensory neurons are further divided into monopolar, bipolar (bd) or multi-dendritic (md). The es and ch organs are monopolar where as dbd and multi-dendrite (md) neurons are multi-dendritic (Merritt and Whittington, 1995). The bipolar dendrite neuron of a stretch receptor organ located along the length of a muscle prevents excessive body wall stretch (Tamarkin and Levine, 1996).

1.3 **Drosophila larval motor neurons**

Motor neurons are located in VNC send out axons to innervate body wall muscle. The region of the axon in contact with muscle is called neuromuscular junction (Keshishian et al., 1996).

**Figure 1.4.**
**Figure-1.4. Image showing larval motor neurons innervating the body wall muscle.** Motor neurons labelled by a pan motor neuron driver, OK371 Gal4>GFP. OK 371 Gal4 by Mahr and Abele, GEP, 2006. Motor neurons of larva originate in ventral nerve cord (indicated by a pink arrowhead) and innervate body wall muscle. Motor neuron labelled in green and muscle in red, pink arrow head points to the brain, white arrowhead points to a motor neuron and blue box encloses body wall muscle of one hemi-segment. Image courtesy, TafheemMasoodi. Scale bar 100 µm.

*Drosophila* muscle fibres are multiply innervated by excitatory motor neurons where glutamate acts as a neurotransmitter(Jan and Jan, 1976; Johansen et al., 1989). *Drosophila* motor neurons in embryonic (Landgraf et al., 1997; Sink and Whittington, 1991) and larval stages (Hoang and Chiba, 2001) were identified. Motor neurons make a stereotyped connection with their target muscles. The organization pattern of larval motor neurons innervating body wall muscles is
known and recognizing the neuromuscular junction allows identification of the motor neurons. The motor axons project into muscle field via two main nerves: the intersegmental (ISN) and segmental nerve (SN) (Figure 1.5). ISN and SN defasciculate at a defined location in the muscle field to innervate their cognate muscles.

**Figure 1.5.**

![Image showing main motor neuron nerves (ISN, SNa-d and TN), innervating larval body wall.](image)

Motor neurons labelled by pan motor neuron driver, OK371 Gal4 driving green fluorescent protein and labelled green. Muscle labelled red using phalloidin conjugate binding to muscle actin. Pink arrow heads point to motor nerves. Scale bar 100 µm.

The segmental nerve (SN) further divide into three different branches, SNa, SNb, and SNc-d, to innervate distinct sets of ventral or lateral muscle. SNa branch innervates lateral muscles, SNbinnervates ventrolateral muscles and the SNc-d ventral muscles (Figure 1.5) (Landgraf and Thor, 2006).

### 1.3 *Drosophila* larval body wall muscle

*Drosophila* larval body wall muscle is classified into three groups: dorsal, lateral and ventral. A schematic representing muscle groups of the body wall hemi-segment is shown in the Figure 1.6: dorsal (blue), lateral (yellow) and ventral muscles (pink). Each of these muscle
groups are innervated by one main motor nerve: dorsal muscle by ISN, lateral muscle by SNa, ventrolateral muscle by SNb and ventral muscle by SNc-d.

Figure 1.6.

Figure 1.6. Schematic of *Drosophila* larval body wall showing dorsal, lateral and ventral musculature. Muscle groups of a larval hemi-segment coloured distinctly; dorsal muscle group blue, lateral muscles yellow and lateral muscles pink and shown as a schematic.

The intersegmental nerve (ISN) projects onto the dorsal muscle field innervating muscles: DA1, DA2, DA3, LL1, DO1, DO2, DO3, DT1, DO4 and DO5 (Figure 1.5 and 1.6); the segmental nerve-a (SNa) projects onto lateral muscles: LO1, SBM, LT1, LT2, LT3 and LT4; the SNb projects onto ventrolateral muscles: VL3, VL4, VL1, VL2, VO1, VO3 and VO2; the SNc projects onto the ventral muscles: VA1, VA2 and VA3 and the SNd projects onto ventral muscles: VO4, VO5 and VO6 (Goodman and Doe, 1993). TN innervates muscle VT1 (Hoang and Chiba, 2001) (Figure 1.5 and 1.6).

1.3 d- *Drosophila* larval interneurons

Interneurons coordinate segmental and intersegmental motor output in vertebrates (Grillner and Jessell, 2009) and invertebrates (Marder and Calabrese, 1996). Interneurons are the main signal
processing elements in the insect brain (Christensen et al., 1993). Interneuron cell body and neurites are confined to VNC (Figure 1.2, cell body coloured blue). The term neurite describes both dendrite processes and axon extensions of a neuron. In *Drosophila* larva, the number of interneuron cell types is approximately equal to the number of neurons, suggesting less redundancy in embryonic interneuron cell types (Rickert et al., 2011). In addition to signal integration (Burrows, 1992; Laurent, 1987), interneurons have a developmental role where early born interneurons in an embryo serve as pioneers to guide and facilitate the organization of motor neurons (Klämbt et al., 1991).

### 1.4 *Drosophila* larval glia

Glia maintains neuronal ion homeostasis (Treherne and Schofield, 1981) by regulating potassium ion concentration and preventing irregular excitation of neurons by removing unused neurotransmitter from a synaptic region (Simard and Nedergaard, 2004). Three main glial types were identified in *Drosophila* larva: 1) surface glia 2) cortex glia and 3) neuropile glia. The first two categories act as blood brain barrier while third class, wraps around individual neurons. Three sub-classes of neuropile associated glia were identified: a) Intersegmental nerve root glia (ISNG) b) Interface glia and c) Segmental nerve root glia (SNG). In larval VNC, intersegmental and segmental glia are closely wrapped around the neurons (Ito et al., 1995). The anatomy of this glia and the extent of wrapping to individual motor neurons are not clear. Wrapping glia associated with the peripheral nerves most likely correspond to ISNG and SNG. Wrapping glia surrounds individual sensory neurons of peripheral nerve is incomplete at first instar but completed at the third instar larval stage (Stork et al., 2008).

### 1.5 *Drosophila* leg neuromuscular system

Adult *Drosophila* is a hexapod uses tripod gait for locomotion, with three legs on the ground (Hughes, 1952). *Drosophila* legs are jointed consisting of coxa, trochanter, femur, tibia and the tarsus.

**Figure 1.7**
Figure 1.7. Image showing *Drosophila* adult motor neurons in the thoracic ganglion. T1, T2 and T3 are neuromeres of thoracic ganglion and AG is the abdominal ganglion. ML is midline and LE is the lateral edge of a hemi-neuromere. Pink arrow points to the motor neuron cell bodies. Figure A, modified from Demerec, 1994.

Movement of leg joints (Figure 1.8) is controlled by the motor neurons located in the thoracic ganglion (Figure 1.7). A total of 14 muscles are present in *Drosophila* leg; innervated by 53 motor neurons from 11 different lineages (Baek and Mann, 2009). The lineage 15 comprises of 30 motor neurons. Motor neurons of lineage 15 innervate along the proximal-distal axis of a leg in a birth order dependent manner. The early born motor neurons innervating proximal regions of the leg (Ltm2 and Tidm) and the late born motor neurons innervating the distal regions (Tirm, Tilm, Talm and Tarm1&2) (Brierley et al., 2009). Primary neuroblasts born during embryonic stages are reactivated in the larva, to generate secondary neurons. The motor neurons derived from a particular reactivated neuroblast remain together in a
cluster known as a lineage. These neurites innervate the leg discs, the future adult leg structures (Truman, 1990).

**Figure 1.8.**

![Diagram showing leg motor neurons innervating musculature of leg segments in Drosophila.](image)

- levator muscle (lm)
- tendon muscle (tm)
- reductor muscle (rm)
- depressor muscle (dm)
- tr - trochanter; fe - femur; ti - tibia and ta - tarsus

**Figure 1.8. Image showing leg motor neurons innervating musculature of leg segments in Drosophila.**

*Drosophila* leg motor neurons labelled by a pan motor neuron driver OK371 Gal4 driving GFP. The muscle of a leg segment is false coloured; levator muscle in brown, depressor in purple, reductor in green and long tendon muscles in blue. The orange coloured arrow head points to the sensory neuron cell body located in the tarsal segment of the leg.

Much of our understanding of arthropod walking comes from the insects like locust, cockroach and stick insect (Burns and Usherwood, 1979; Delcomyn et al., 1973). In forward locomotion, foreleg anchors to the ground while middle and hind legs promote the movement. The mid and hind tarsi always placed on the ground behind coxa to push the body forward by the extension of the tibia on the femur (Hughes, 1952). A pair of antagonistic muscles, depressors-
coloured purple and levators-coloured brown, in figure 1.8 play a key role in locomotion. The levator muscle facilitates extension or to lift while the depressors lower the legs (Delcomyn, 1988). The activity of levator-depressor muscles located in different segments of the leg results in the protraction (movement of a body in anterior direction) or retraction (movement of a body in posterior direction) of the body (Demerec, 1994; Soler et al., 2004). For example, the levator muscle of trochanter become active while lifting the foot off the ground and the depressor muscle before the foot placed on the ground.

With this introduction, results will be explained in the following chapters. Chapter 3 contains detailed procedures of cloning of *DVGlut* enhancers, generation of transgenic flies and analysis of expression patterns in the generated transgenic flies to identify small sets of labelled neurons. This chapter also discusses implications of multiple enhancers providing expression in a common set of neurons. Chapter 4 includes the description of anatomy and the organization of identified motor and interneurons in the larva. In this chapter, the function of one specific class of glutamatergic interneurons in larval locomotion is described. Chapter 5 discusses the specific organization of larval neurons and probable underlying mechanisms in larval body wall bending.