SYNOPSIS
Background:

Sleep is a vital behavior that consumes one-third time of human life. The sleep-wake cycle found in mammals including humans, show a periodicity in the physiology and behavior of nearly 24 hours. This is in synchrony with environmental day and night cycle. Periodicity showing a rhythm of nearly 24 hrs is referred to as 'circadian rhythm'. This rhythm of sleep & wakefulness is not restricted to only mammals but is well exhibited in many other organisms like single cell prokaryotes to humans. In higher eukaryotes like fruit flies (Drosophila melanogaster) to humans it is a function of adult central nervous system. It also shows significant homology in the structure & function of the genes involved in this pathway, in these organisms. In reality sleep-wake cycle of humans is similar to the rest-activity cycle of D. melanogaster. Thus Drosophila provides a novel model system to understand the molecular nature of sleep and biological clock of human. Biological clock is a function of pacemaker clock cells that have an auto regulatory molecular feedback pathway generated by certain core clock genes. It is a function of few pacemaker neurons demarcated in the adult nervous system for this purpose, in these higher eukaryotes.

There are many sleeping disorders that result due to the disbalance in normal sleeping cycle. Excessive day time sleepiness, hypersomnia, insomnia, sleep apnea, narcolepsy, restless leg syndrome, parasomnia, periodic limb movement disorder, obstructive sleep apnea and central sleep apnea are a few of such disorders. Sleeping abnormalities affect number of behavioral and psychological rhythms like alertness of mind and body. To understand sleeping disorders the molecular basis of sleep-wake cycle is required to be explored. This will help to find cure to many sleep related neurological disorders in the long run.

In this study we have tried to understand the regulation of sleep by probing the molecular control of period gene, a core component of circadian pathway. To address this question we have adopted a series of genetic and molecular
experiments in *D. melanogaster*. We have also carried out some preliminary study to establish fruit fly as a model to carry out a screening system for drug development against different sleep disorders.

**Rationale:**
The sleep wake cycle is a manifestation of the circadian rhythm. The circadian cycle is comparatively better understood at the molecular level, as documented by some recent and rapid developments in this field. Core clock genes in the nucleus & cytoplasm of pacemaker neurons control endogenous circadian clock by forming intracellular molecular negative feedback loops. The network of such clock cells generates and maintains the biological clock in these organisms. The *period* (*per*), a transcription factor is one such core clock gene. In both mammals (including humans) and fruit flies, it controls the daily rhythms, by interacting with other core clock molecules. The gene in itself shows a robust daily rhythm in its transcript and protein expression temporally and spatially, in the pacemaker neurons.

Based on earlier studies, the regulation of *period* can be classified into two steps: transcriptional control in the nucleus and the post-translational regulation in the cytoplasm. These regulations control a feedback loop at different steps so as to have a precise rhythmicity of nearly 24 hours both in its amount and compartmentalization. Elimination of both these regulations still shows a rhythmicity of lower amplitude. This suggests a possibility that another regulation exists at the post-transcriptional stage that adds to the robustness of the cyclicity. Although till date there is no experimental proof supporting this. This project was started to find any such additional mode of regulation so as to bridge the gaps in the feedback loop.

In many organisms RNA-mediated gene regulation plays an important role in epigenetics and genome control. In the process the RNA of the gene regulates its own expression either by establishing the state of chromatin, or by meddling in its mRNA stability either by its degradation or translational inhibition. Such RNA processing methods regulating gene expression, based on the mechanism of complementary base pairing is referred broadly as RNA interference (RNAi) in
animals. In this work, we have tried to explore the possibility of the involvement of such kind of a regulation in the per gene regulation. That might fine-tune the temporal & spatial expression of the gene, thereby maintaining the feedback mechanism at the molecular level.

**Part I: Molecular mechanism of circadian rhythm in wild type Drosophila melanogaster**

To determine the molecular basis of per in the pacemaker neurons, which might be responsible for the residual rhythmicity, we examined the periodicity of the sense per transcript in these tissues by performing semi quantitative RT-PCR using cellular RNA isolated from the head of wild type flies. We also did strand specific RT-PCR for per RNA to find the existence of any other transcripts in this locus. To our surprise we indeed found a transcript in the reverse orientation showing rhythmic expression, which is not reported till date for D. melanogaster. Later it was found that the antisense transcript corresponding to per gene is restricted to only a portion of the gene corresponding to the PAS2 domain generating sequence by 5’ and 3’ RACE (Rapid Amplification of cDNA Ends) experiments. The presence of the per sense and anti sense transcripts, was established by RNA in situ experiments, in the adult brains of wild type flies. As other group of small RNAs are found in vivo in many instances where antisense RNA is found, small RNA Northern experiments were performed that showed the existence and accumulation of a special species of small RNAs corresponding to the per locus.

**Part II: Role of anti sense per transcript in the per-tim feed back loop in pacemaker neurons of Drosophila melanogaster.**

Once we found the existence of an antisense transcript corresponding to part of the per gene locus in D. melanogaster, the next question was to find out the role of such a transcript in the circadian feed back loop. For this we examined the role of mutations of RNA silencing factors, on locomotor activity rhythm of wild type fruit flies. We found a marked difference in the activity rhythm between the wild
type and mutant flies. Strand specific RT-PCR for the *per* sense and antisense transcripts in these mutants were performed. The expression of *period* small RNA was detected in these mutants by northern hybridization. To verify whether the mutational effect of RNAi factors is only confined to the RNA transcripts or is evident in the protein accumulation as well, the expression of Green Fluorescent Protein (GFP) driven by *period* promoter in adult brains using the confocal microscopy was carried out. The mutations affect protein expression pattern both spatially & temporally. Immunostaining against PER in heteroallelic mutant background confirmed changes in expression pattern of the PER protein in the pacemaker neural cells of the adult brain in *D. melanogaster*. These results together, proposes an unexpected contribution of RNA silencing in the control of *period* gene, which is required for fine-tuning the expression & accumulation of the *per* gene product.

**Part III: Neurotransmitter receptors in the sleep-wake pathway**

As previously reported, the physiology & behavior of daily rhythm is a product of the molecular circadian feedback loop. The feed back loop as a core clock regulates the phenotype through a plethora of down stream genes known as Clock Controlled Genes (CCGs). Many of the neurotransmitters are such CCGs that help in transmitting the clock generated signals so as to finally synchronize the internal cell environment to the external physical environment.

Though many such neurotransmitters are the target of various sleep enhancing and repressing drugs, very little is known about the mode of action of these drugs on these neurotransmitters. Our initial work in *D. melanogaster* on some of these receptors unravels a vast possibility of using fly as a model organism for screening the characteristics and effectiveness of such drugs. By comparing the rhythmic activity of wild type versus mutant flies for some neurotransmitter receptors we have tried to identify whether any of these receptor genes under study, do have a role in the sleep wake cycle of the fruit fly.
Significance:
We have identified a novel post-transcriptional regulation of per gene by the production of antisense transcript, as a new dimension in the regulation of core clock gene period, required for maintenance of the negative feedback loop. This regulation is also manifested as a molecular fine tuner of phenotypic behavior in Drosophila rest-activity cycle. Several transcriptional and post transcriptional controls work in concert to deliver the proper regulatory dynamics of rapid ups and downs of period transcript levels in the 24 hour time frame. Lastly, identification of neurotransmitter receptors effective on the normal rest-activity cycle in Drosophila may provide a platform for pharmacogenomic and toxicogenomic testing of different drugs to find cure for various sleep disorders.