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Inwardly Rectifying Potassium Channels in *Drosophila* Regulate the Sleep/Wake Behaviour through PDF-Neurons

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ABSTRACT

Potassium channels are important modulators of cell function depending on the cell type of where they are expressed. They are involved in regulation of cell membrane resting potential, potassium homeostasis and control a variety of cellular functions including metabolism. In this study we determined that a regulator of *Pigment dispersing factor*, PDF-immunoreactive neurons in the *Drosophila melanogaster* adult brain, is an inwardly rectifying potassium channel, IRK1. Knocking down the potassium channels specifically on PDF expressing neurons using UAS-GAL4 RNA^{i} system resulted in altered axonal projections of lateral neurons (LNv) towards the dorsal neurons (DN). Moreover, it was observed that lack of the potassium channels also caused a robust increase in sleep and reduction in the fly's active period during the day. We observed that the normal circadian control of the morning and evening anticipation is also dependant on these potassium channels. The flies deficient in IRK1 channels didn't show an evening anticipation peak. Another interesting disclosure during this study was the inability of PDF-Tri neurons to undergo programmed cell death in the absence of inwardly rectifying potassium channels. Hence, IRK1, though poorly expressed in the *Drosophila* central nervous system, plays an important role in the normal functioning of PDF expressing neurons. Further studies are needed to elaborate the physiological roles of *Drosophila* potassium channels which may lead to a better understanding of human Kir channels related to pathological conditions and diseases.

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Authors' Contributions

MA conceived and designed the study, performed the analysis and wrote the article. NR helped in designing and proof reading the manuscript.

Key words

PDF neurons, Inwardly rectifying potassium channels, Sleep/wake cycle, Kir channels, PDF-Tri neurons.

INTRODUCTION

Sleep is general from the insects to the mammals. The fruit fly *Drosophila melanogaster* manifests all behavioural characteristics of the mammalian sleep (Hendricks *et al*., 2000; Shaw *et al*., 2000). Therefore, we used *Drosophila* as a genetic model for the identification of the novel genes which modulate sleep behaviour. Sleep wake cycle is not controlled by the few specific genes or a single locus (Cong *et al*., 2015). Studies showed that it is under the control of the two main regulatory mechanisms, like the circadian system which drives the sleep with the periodicity of 24 h and the homeostatic system which makes sure that sufficient amount of the sleep is obtained. The circadian rhythms are the biological cycles which are set through the internal timing mechanism (Nagoshi *et al*., 2009).

In the brain of *Drosophila*, the circadian cells have ~150 clock neurons. Past research has shown that PDF positive ventrolateral neuron (LNVs) of the circadian neuronal system is very crucial for the regulation of sleep by promoting the wakefulness (morning activity) (Parisky *et al*., 2008) while the 5th LNv, dorso-lateral neurons (LNds) and the dorsal neurons (DN1s) coordinate the evening activity and also modulate the suppression of sleep during the starvation (Keene *et al*., 2010).

In all animals including *Drosophila*, the flow of information in the nervous system is through the changes in the resting membrane potential created across the neuronal membranes through ion channels. *Drosophila* genome express almost 150 ion channels which are clearly related to 350 human ion channels (Ashcroft, 2006; Cannon, 2006; Hodge, 2008). Inwardly rectifying potassium channels are responsible for various functions including cellular metabolism and transport of potassium ions. Their functions are highly dependent on the cell type where they are expressed (Luan and Li, 2012).

The availability of *Drosophila* whole genome sequence enabled the recognition of previously unrecognized ion channels. A subset of specialized inwardly rectifying potassium channels (Kir) had been identified in the *Drosophila* genome, which encodes three cDNAs dKirI, dKirII and dKirIII. The expression of dKir

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channels varies greatly during fly developmental process (Doring *et al*., 2002).

dKirI is highly expressed in larval/adult midgut, malphigian tubules and salivary glands, where it is important for potassium homeostasis. *In situ* hybridization reveals the localization of these channels in the tubule and the ureter where they perform both reabsorption and secretion of primary urine (Evans *et al*., 2005). Moreover, dKirI has been found to be poorly expressed in the fly brain, but is enriched in ventral lateral neurons (LNv) (Nagoshi *et al*., 2009; Kula-Eversole *et al*., 2010). It has not been investigated previously what role these channels play in the fly circadian rhythms. Latest studies using mutant knock out model organisms for Kir channels might not have revealed many important physiological functions of these channels.

dKirI is most closely related to human Kir2 family based on homology and shows profound similarity (Doring *et al*., 2002). Human Kir2 family is the most diverse group of ion channels which are responsible for neuron excitability and plasticity in various organs. The identification of potassium channel modulators is of considerable importance in both understanding the physiology and identifying therapeutic targets in various disorders (Abraham *et al*., 1999).

This study aims to elucidate the physiological role of dKirI channels present on the clock neurons, which can serve as a step forward towards a better understanding of the specific role of each clock neuron in the physiological difference of morning and evening clocks.

METHODS

Fly stocks were received from the Bloomington Stock Center (Bloomington, Indiana, USA; http://flystocks.bio.indiana.edu/). All flies, unless otherwise stated, were maintained on enriched Jazz mix standard fly food (Fisher Scientific, Gothenburg, Sweden) supplemented with yeast extract (VWR, Stockholm, Sweden). Flies were maintained at 25ºC in an incubator at 60% humidity on a 12:12 light:dark cycle. For the experiments male flies were collected soon after eclosion and maintained on normal food for 5-7 days before the experiments were performed.

Flies crossed to GAL4 drivers (GAL4 drivers crossed to UAS-RNAi lines) and controls (GAL4 drivers or UAS-RNA^{i} crossed to w1118) were raised at 25ºC until the adults emerged (F1 generation); once collected adults were raised at 29ºC for the appropriate times. All assays were performed at 25°C. In all assays, the GAL4 drivers and UAS transgenic flies were crossed to w^{1118} flies and their F1 progeny used as controls.

For fly brain immunohistochemistry, male flies were beheaded and kept in 1X PBS (phosphate buffer saline) for 15 min. The heads were then transferred to 1 ml of 1X fix buffer (0.2 mM Sodium Phosphate buffer, 10 mM EGTA, 4 mM $MgCl_2$) and 4% paraformaldehyde (PFA) overnight at 4ºC. After fixation, the heads were transferred to a silicon padded petri dish and brains were dissected in 1X PBS buffer using a dissection microscope and fine forceps. Brain tissue was fixed in 4% PFA and 1X PBS for 2 h. It was then blocked with 400 µl of 1% goat serum (Abcam, Cambridge, UK) in PBL (Phosphate Buffer with Lysine) overnight and again with 5% goat serum for 1 h. The brains were then treated with primary antibody (anti-PDF mouse monoclonal with a dilution of 1:1000 in 0.1 M PBL containing 0.5% Triton X-100) and incubated on rotation at 4ºC. Following 18 h of incubation the brains were washed three times with 1% goat serum and then treated with secondary antibody (ThermoFisher Scientific, MA, USA, goat anti-rabbit Alexa Fluor 594), diluted 1:2000 in 0.1M PBL, overnight. The tubes were covered with aluminum foil during the whole process. Tissues were washed with 1X PBS containing 0.5% Triton for 3 h and then treated with DAPI (1:1500) for 15 min. After washing the DAPI with 1X PBS twice, the brains were mounted in Vectashield (Vector Laboratories, Peterborough, UK). Images were captured on a Zeiss LSM 710 confocal microscope and visualized using ImageJ.

For starvation assay, the *Drosophila* activity monitoring system (DAMS) from TriKinetics was employed to analyse the starvation resistance and activity/sleeping index in our experiments (Hergarden *et al*., 2012). Flies were placed into isolated tubes with a diameter of 5 mm. As described previously, the system checks the flies' beam-crossing numbers (Pfeiffenberger *et al*., 2010). Afterwards, the survival time and activity can be calculated and measured, respectively.

For activity index and sleep studies *Drosophila* activity monitoring system was employed. Thirty-two male flies from control and experimental flies, aged 5–7 after eclosion, were placed in the tube as described above. Normal food was used to replace agarose. The activity and sleep behaviour of a 24-h period under 12:12 LD cycle was recorded by the *Drosophila* Activity Monitoring System (DAMS) automatically and then analysed using GraphPad Prism v5.03. For the study of circadian rhythms three continuous days of dark and three consecutive days of light were provided in the DAMS to the 5-7 days old flies. The data collected were analysed using FaasX to draw eduction graphs.

All the statistical analyses were performed using GraphPad Prism v. 5. Student's T-test was performed to compare the control and mutant samples.

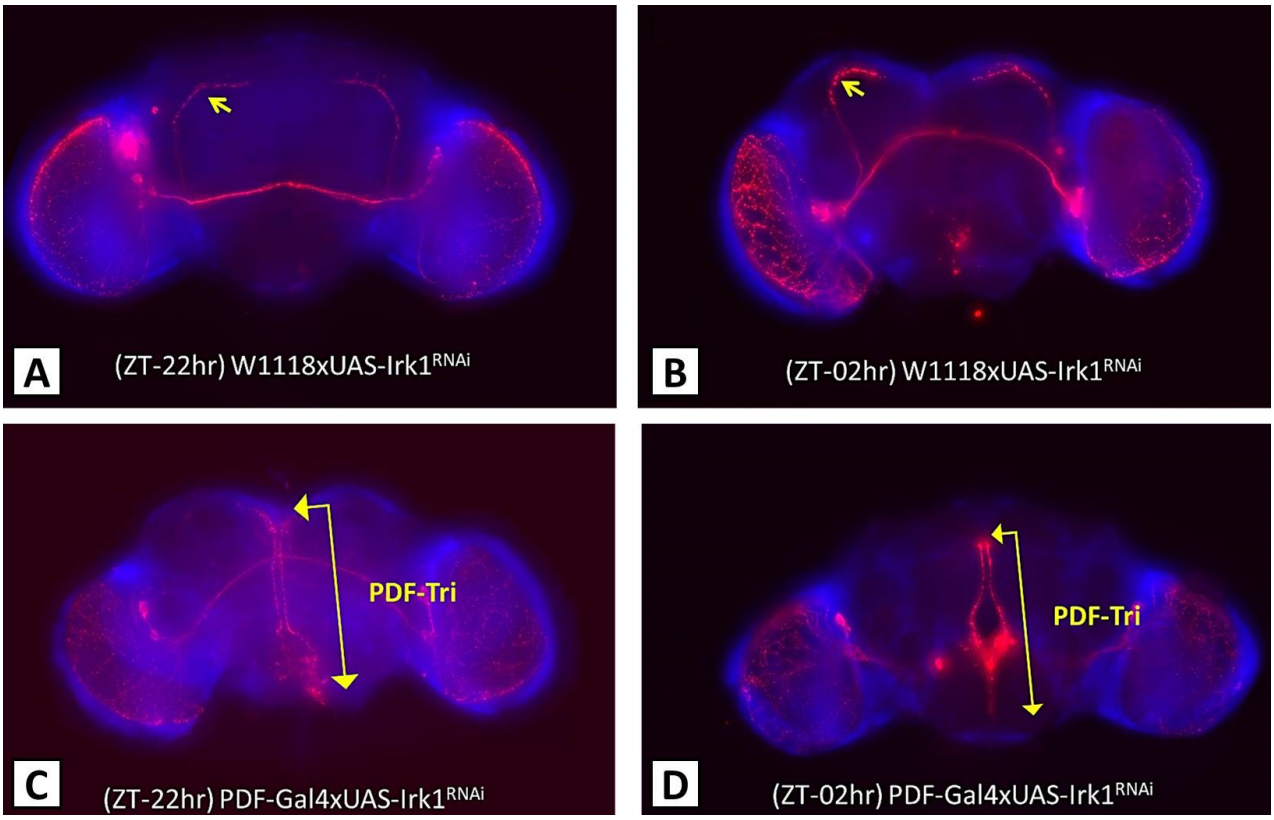

Fig. 1. PDF-expressing neurons show a different arborisation pattern in the absence of IRK1 channels. Male flies aged 5-7 days raised on normal food and12:12 LD cycle were beheaded at two different time points. The brains were dissected and stained with anti-PDF antibody (red). A and B, the control flies with sLNvs sending projections towards the dorsal part of the brain (indicated by the yellow arrow) both before and after the lights were turned on. C and D, the IRK1 knock down flies where axonal projections towards the dorsal neurons were absent. The yellow double arrow indicates the presence of PDF-Tri neurons, which are usually degenerated after few days of eclosion. ZT-22 h indicates two hours before the lights were turned on (ZT 0 h = 0800 h), ZT-02 h indicates two hours after the lights were turned on.

RESULTS AND DISCUSSION

Knocking down Irk1 ion channel cessate PDF signalling

Circadian clocks play a critical role in an organism's normal physiological functioning (Takahashi *et al*., 2008). They operate in three stages; the core clock, input pathways and the output pathways but do not act mutually exclusively (Allada and Chung, 2010). In *Drosophila* there are 150 clock neurons, which are part of the neural network governing the circadian behaviour and can be divided into further subsets (Nitabach and Taghert, 2008). The pigment dispersing factor is an important effector of the 20 ventral lateral neurons (LNv). It has been known that the smaller subgroup sLNv send projections towards a number of neural circuits including, the dorsal lateral neurons (DN), mushroom bodies (MB) and pars intercerebralis (PI), which are the critical regulators of fly sleep/wake cycles (Pitman *et al*., 2006; Joiner *et al*., 2006). In order to see any differences in the projection pattern of PDF-neurons lacking potassium channels IRK1, we specifically knocked down the inwardly rectifying potassium channels on the PDF expressing neurons using the RNA interference system in flies. The male progeny was collected and flies were raised on standard fly food with 12:12 light dark cycle. The brains of 5-7 days old flies were dissected and stained with anti-PDF antibody. The flies were beheaded at two time points; ZT 22h (two hours before the lights were turned on) and ZT 2h (two hours after the lights turned on) as shown in Figure 1. Interestingly, it was observed that in the IRK1 knockdown flies, the LNvs do not show any axonal projections towards the DN cells in the dorsal part of the brain (Figure 1; C and D indicated by the arrow), which was otherwise seen in the controls as shown in Figure 1A and B. This phenomenon was consistent before and after the lights were turned on as can be seen in Figure 1C and D.

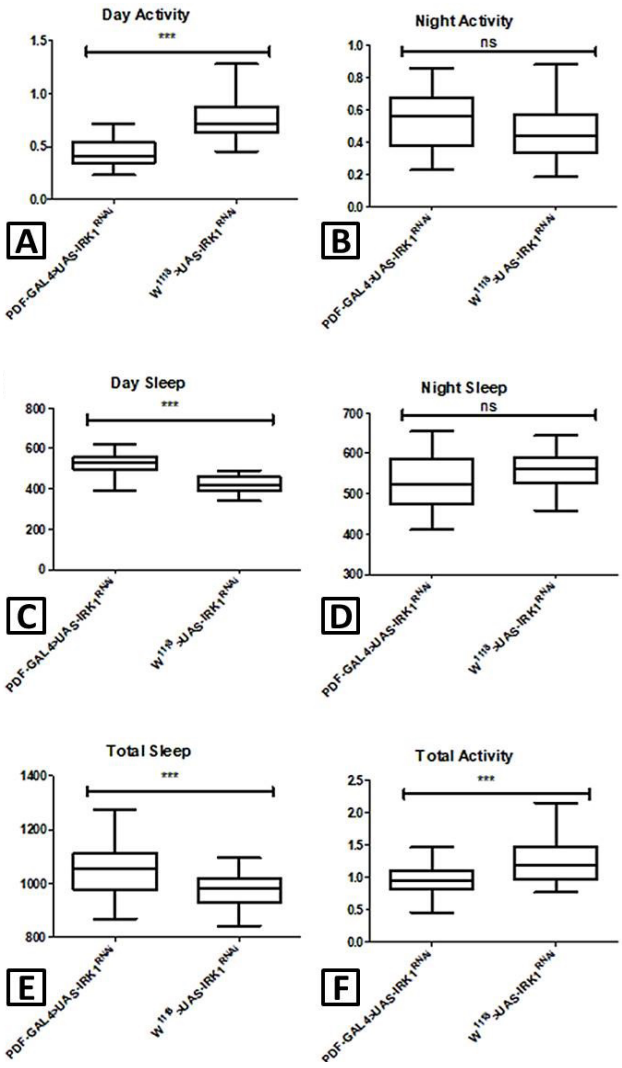

Fig. 2. IKR1 knock downs show an increase in day sleep and decrease in activity. 5-7 days old flies raised on normal food and 12:12 LD cycle were put in DAMS and observed for consecutive 7 days under the same conditions with 40-60% humidity. The data was analysed for sleep and activity. **A and C**, significant decrease in day activity and increase in day sleep, respectively. **B and D**, no effect on night activity and sleep. **E and F**, the total change in activity and sleep over the period of 7 days. Error bars show standard error of the mean (P<0.0001).

PDF-Tri neurons do not undergo apoptosis without IRK1 channels

Another interesting observation is the retention of PDF-Tri neurons in the Irk1 knock down flies (Fig. 1C, D). These neurons appear transiently during the adult ecdysis starting from the mid-pupal stage (Selcho *et al*., 2018). However, later they undergo programmed cell death within few days of eclosion. PDF-Tri neurons are the non-clock, PDF expressing neurons believed to be involved in the processes active during the transition from pupa to the adult (Gatto and Broadie, 2011; Helfrich-Foster, 1997). There has been recent studies as well in *Drosophila* related to proteins involved in cell proliferation and apoptosis (Zhang *et al*., 2016)

Loss of inwardly rectifying potassium channels leads to increased day sleep

There is evidence that suggests that the specific subsets of LNv expressing PDF, including the so-called "evening", or "E", cells and DN1 cells are important for the morning activity under LD condition (Storleru *et al*., 2007; Zhang *et al*., 2010a, b; Guo *et al*., 2014). Next, we determined if this altered arborisation pattern due to loss of potassium channels is affecting the sleep/wake behaviour of the fly. DAMS were setup to determine if the amount of overall sleep is changed. 5-7 days old male flies were put in DAMS tubes with normal fly food. The flies were observed for 7 consecutive days with normal 12:12 light dark cycle and 40-60% humidity. The data was analysed for the amount of sleep and activity recorded with reference to the beam crossings per minute. It was observed that the flies lacking the potassium channels on the PDF neurons showed a significant increase in day sleep and subsequent decrease in day activity (Fig. 2A, C). This resulted in an overall increase in the sleep of the mutant flies lacking the inwardly rectifying potassium channels (Fig. 2E, F). Apparently there was no effect observed on the night sleep and activity as shown in Figure 2B and D. As DN1 cells are acting as relay point between the sLNv and clock output, the absence of PDF signalling leads to misregulation of sleep in flies.

Inwardly rectifying potassium channels an effector of the circadian rhythm

It is known that the PDF neuropeptide expressing (LNv) are especially important for morning anticipation and called the morning (M) cells, while dorsal lateral neurons (LNd) and dorsal neuron (DN1) underlie evening anticipation and are called evening (E) cells. This pacemaker network is considered the most extensively studied CNS network but there are new revelations underway as well (Kunst *et al*., 2014; Guo *et al*., 2016). The anticipation of the morning and evening is largely dependent on the PDF expressing neurons in the central brain. To determine if the IRK1 channels play a role in maintaining the circadian rhythm, we set up the DAMS again with three days of light and three days of dark cycle. The data collected was analysed using FaasX. The average group activities were plotted in the form of eduction graphs (Fig. 3). It was observed that the controls have

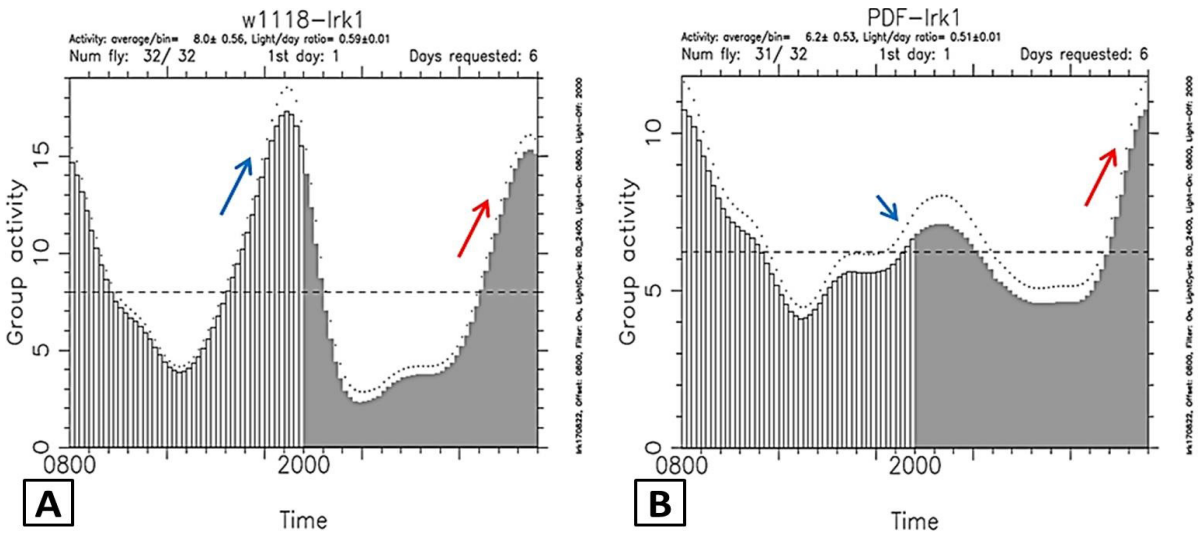

Fig. 3. Knocking down IRK1 causes dampening of evening anticipation peak. Male flies aged 5-7 days raised on normal food and 12:12 LD cycle were put in DAMS under DD: LL condition to observe the circadian rhythm. The flies were left for 24 hrs in DAMS to acclimatize to the environment. First three were continuous dark and last three days were continuously light exposed. The average group activities were plotted as eduction graphs using FaasX. On the x-axis time is plotted in hours and on the y-axis are the average group activities of flies in one channel. **A**, the control flies with normal evening and morning anticipation peaks as indicated by the blue and red arrows, respectively. **B**, the diminishing evening peak (blue arrow) and the normal morning peak (red arrow).

normal morning and evening peaks (Fig. 3A); however, the evening anticipation peak in the mutant flies lacking the potassium channels was significantly dampened (Fig. 3B). It is already known that under DD condition, sLNv regulate both the speed and behaviour of PDF dependant clock neurons. This suggests that absence of PDF signalling is followed by some or all DN1s or DN3s malfunction (Yao and Shafer, 2014).

CONCLUSION

The list of sleep regulators and their implications is ever expanding. The present study aimed towards elucidating the role of potassium channels in regulating the sleep/wake rhythm of *Drosophila*. We determined that IRK1 is an important regulator of PDF expressing neuron excitability and its output clock. Moreover, the programmed cell death of PDF-Tri neurons is halted in the absence of these inwardly rectifying potassium channels. Although the PDF neuronal network is the most broadly studied clock system, there are still gaps that need to be filled in. The enrichment of LNvs with Ir transcripts indicates an important future goal for circadian neuron function (Kula-Eversole *et al*., 2010).

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Statement of conflict of interest

The authors declare no conflict of interest.

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