Immune stimulation reduces sleep and memory ability in *Drosophila melanogaster*

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Abstract

Psychoneuroimmunology studies the increasing number of connections between neurobiology and immunology. We establish *Drosophila melanogaster* as a tractable model in this field by demonstrating the effects of the immune response on two fundamental behaviours: sleep and memory ability.

We used the Geneswitch system to upregulate peptidoglycan receptor protein (PGRP) expression, thereby stimulating the immune system in the absence of infection. Geneswitch was activated by feeding the steroid RU486, to the flies. Importantly, by stimulating the immune system of adult flies in the absence of infection we have avoided the added complications of developmental and disease effects that have confounded other studies. We used an aversive classical conditioning paradigm to quantify memory and measures of activity to infer sleep.

Immune stimulated flies exhibited reduced levels of sleep, which could not be explained by a generalised increase in waking activity. The effects on sleep were more pronounced for day compared to night sleep. Immune stimulated flies also showed a reduction in memory abilities.

These are important results as they establish *Drosophila* as a model for immune-neural interactions and provide a possible role for sleep in the interplay between the immune response and memory.

Keywords

immune-neural interactions, imd, geneswitch, PGRP-LCa

Introduction

Psychoneuroimmunology, in vertebrates, studies the connections between neurobiology and immunology[1]. In invertebrates, immune response negatively affects learning and memory in bees[2]. A tractable invertebrate model of these immune-neural links would provide a fillip to this field[3]. The fruit fly, *Drosophila melanogaster*, has been tremendously helpful to the analysis of associative learning[4] and immunity[5]. In this paper we demonstrate immune-memory links in *Drosophila* and further expand the paradigm by showing immune-sleep interactions in flies.

Sleep is a resting state where the sleeper exhibits inattention to the environment and is usually immobile[6]. *Drosophila melanogaster* like vertebrates have been shown to have a distinct sleep state. In flies, a sleep episode is defined as a period of immobility lasting five minutes or longer[7,8]. Such intervals are associated with reversible increases in arousal threshold, which can be further augmented following sleep deprivation[9], are associated with changes in brain electrical activity[10], and are reduced by several drugs like caffeine and modafinil and are increased by antihistamines[7,8]. As in mammals, sleep deprivation leads to a rebound in quantity of sleep[8].

Infections increase sleep in humans, most likely through induction of proinflammatory cytokines[11]. Similarly, Kuo infected flies with gram-negative bacteria, leading to an increase in sleep[12]. On the contrary, Shirasu-Hiza infected flies with gram-positive bacteria and observed that they slept less[13]. The latter agrees with findings of increased immune gene transcription and resistance to disease in sleep-deprived flies or in reduced sleep phenotype transgenic flies[14,15].

Experiments, looking at the effect of immunity, often confound the disease used to stimulate the immune system and the immune response itself. Here, we activated the immune system non-pathogenically. We used Geneswitch[16] to up-regulate peptidoglycan receptor protein LCa (PGRP-Lca) in adult flies. PGRP-Lca is a pattern recognition protein that recognizes gram-negative bacteria, setting off the IMD immune pathway and leading to the expression of antimicrobial peptides[17]. Geneswitch is activated in the presence of the steroid RU486. We used an aversive classical conditioning paradigm to measure memory abilities of flies[18]. Sleep was measured using the *Drosophila* Activity Monitoring System 2 (DAMS2, Trikinetics, Waltham, MA).

Methods and Materials

The Geneswitch line w^{1118};P{w$^{+mW.hs}$=Switch1}$bun^{Switch1.32}$ (hereafter referred to as *GS1.32*) drives expression of RU486-activated GAL4 in adult fat bodies[17] (http://flystocks.bio.indiana.edu). The three genotypes used were GS1.32>PGRP-Lca(w^{1118};*GS1.32*/+; *UAS-PGRP-Lca*/+), and the control genotypes GS1.32/+(w^{1118}; *GS1.32*/+; +/+) and +/PGRP-Lca (*+/+;UAS-PGRP-Lca/+)*.

Flies were maintained under standard conditions. Males and females were selected at eclosion and flies were 1–3 days old at the beginning of the experiment. Both sexes were used for the memory assay. As is common in fly research, only males were used for the sleep assay due to their more extreme phenotypes[19].

Geneswitch

RU486 was added to *Drosophila* food (200 μM final concentration). For the memory assay, flies were fed for two days with RU486 before the start of the training and returned to the RU486 food after training. For the sleep assay, flies were placed in vials containing RU486 food for two days to allow feeding. After two days flies were immediately loaded into tubes containing more of the RU486 food. For all lines we have flies fed with RU486 and genetically identical animals cultured on fly medium supplemented with an equal amount of vehicle (80% ethanol) that lacked RU486.

Memory assay

Each sample was a single sex group of 50 adult flies. Conditioning consisted of 5 training sessions separated by 20min intervals. In each training session flies were first

104 exposed for 30s to one odorant simultaneously with mechanical shock delivered every
105 5s. This period was followed by a 60s rest period (no odour and no shock). Then, for
106 30s another odorant was delivered, without shock. Flies were either conditioned against
107 3-octanol or 4-methylcyclohexanol (both 0.6ml/l of paraffin).

108 24 hours after the conditioning period flies were transported to the choice point of a T-
109 maze, where they were allowed to choose between the two odors for 60s. The memory
110 score was the proportion of individuals choosing the correct odour, i.e. not the one they
111 were trained against. One hundred and fifteen replicates were carried out, distributed
112 between the genotype, sex, RU486 (presence/absence) and odour used.

113

114 Sleep assay

115 Locomotor activity was monitored by DAMS2, at 25°C, continuously for seventy-two
116 hours under a 12:12 light:dark cycle. Output from DAMS2 was the number of times a
117 fly crossed an infrared beam in a 1min period (bin). 384 flies were tested, divided
118 between genotype and RU486 (presence/absence).

119

120 Data analysis for sleep assay

121 The DAMS2 output was converted to three measures; 1) Sleepbins per hour: number of
122 minutes when a fly is asleep in an hour, 2) Mean waking activity: the mean activity
123 taking into account only those bins that are classified as ‘waking’ and 3) Bouts of sleep:
124 the number of sleep episodes.

125 Flies sleep differently during the day and night[20]. Therefore for each dependent sleep
126 variable we ran two ANOVAs one for day and one for night. The independent variables

were genotype and RU486 (presence/absence). The important term here is an interaction term between genotype and RU486. If this was significant, we could say the genotypes responded differently to the treatments. But we still would not know which genotype's response is different from which. It is quite possible that the two control genotypes' responses to treatment are different from each other but not statistically different from that of the immune stimulated genotype. This would be uninteresting. Therefore if the interaction term was significant here, I repeated this analysis twice, once for genotypes GS1.32>PGRP-Lca vs GS1.32/+ and once for genotypes GS1.32>PGRP-Lca vs +/ PGRP-Lca. If the interaction terms in both these ANOVAs are significant I can say that genotype GS1.32>PGRP-Lca (the immune stimulated genotype) responses differently to the control genotypes in either day or night. Using a Bonferroni correction the significance level α was reduced to 0.0083 (0.05/6). All analysis was carried out using STATA12.

Zone of inhibition assay

This assay measures antibacterial activity: it is based on the ability of immune proteins to inhibit bacterial growth when placed onto an agar plate seeded with bacteria (*Arthrobacteur globiformis* 125µl of an overnight culture per 50ml of agar). Thirty seven GS1.32>PGRP-Lca flies, 17 fed RU486 and 20 not fed RU486 were used. Each fly was homogenized in 30µl of ringer solution. Five microlitres of the supernatant from the centrifuged solution (1300g for 10 min at 4°C) were pippetted into a hole on the agar plate. This was incubated for 48hrs (30°C). The resultant ZOI were measured as the mean of three diameters.

Results

Feeding RU486 to GS1.32>PGRP-Lca flies increased their antibacterial activity by 26% ($t = -2.3263$, $df = 29.202$, $p = 0.02715$).

Immune stimulation effects on memory

Genotype had a significant effect on memory score ($F_{2,109} = 22.46$, $p < 0.0001$). Neither sex, whether RU486 was used, nor odour used had a significant effect on memory score. GS1.32>PGRP-Lca flies, showed a 11.4% decrease in memory scores when fed RU468 relative to those not fed RU468 of the same genotype (interaction between genotype and RU486 was significant $F_{2,109} = 5.76$, $p = 0.0042$). See Figure 1. As feeding RU486 to GS1.32>PGRP-Lca flies leads to an increased immune response, immune stimulation decreases memory scores.

Immune stimulation effects on sleep

Immune stimulated males (GS1.32>PGRP-Lca fed with RU486) showed a 23% decrease in sleep during the day relative to controls (GS1.32>PGRP-Lca vs GS1.32/+: $F_{1,4607} = 136.29$, $p < 0.00001$, GS1.32>PGRP-Lca vs +/ PGRP-Lca: $F_{1,4535} = 26.87$, $p < 0.00001$) and a 9% decrease at night (GS1.32>PGRP-Lca vs GS1.32/+: $F_{1,4607} = 85.53$, $p < 0.00001$, GS1.32>PGRP-Lca vs +/ PGRP-Lca: $F_{1,4535} = 8.49$, $p = 0.0036$). See Figure 2. There was no corresponding change in mean waking activity during the day ($F_{2,6839} = 0.5$, $p = 0.6044$), or during the night (GS1.32>PGRP-Lca vs GS1.32/+: $F_{1,4607} = 63.34$, $p < 0.00001$, GS1.32>PGRP-Lca vs +/ PGRP-Lca: $F_{1,4535} = 1.96$, N.S.). There was no change in the number of sleep bouts during the day (GS1.32>PGRP-Lca vs GS1.32/+: $F_{1,4607} = 6.42$, $p = 0.0113$, GS1.32>PGRP-Lca vs +/ PGRP-Lca: $F_{1,4535} = 10.43$, $p = 0.0012$) and a small but significant increase (0.5%) at night (GS1.32>PGRP-

173 Lca vs GS1.32/+: $F_{1,4607} = 16.38$, $p < 0.00001$, GS1.32>PGRP-Lca vs +/ PGRP-Lca:

174 $F_{1,4535} = 7.56$, $p = 0.0060$).

Discussion

Adult flies, which are immune stimulated but are secluded from the confounding effects of infection, exhibit reduced levels of sleep both during day and night. Immune stimulated flies have slightly more fragmented sleep at night, as evinced by an increase in the number of sleep bouts. The same immune stimulation also leads to a reduction in memory abilities.

The reduction in sleep cannot be explained simply in terms of a generalised increase in activity. Stimulating the immune response had no effect on mean waking activity during the day or night, but these flies slept less than the non-stimulated controls.

Our sleep results agree with a previous study by Shirasu-Hiza showing a similar outcome after gram-positive bacterial infections[13]. However Kuo[12] found that when they infected flies with gram-negative bacteria, the flies slept more. The discrepancies in sleep were explained by Kuo *et al*. as being due to different types of infection. Our work did not use an infection but rather a direct stimulation of the immune response. By upregulating PGRP-Lca we reproduced the immune response associated with gram-negative bacteria. This suggests that if type of infection were the cause of the discrepancies, our results would have just mirrored those of Kuo et al. The two previous experiments were different in numerous other methodical aspects, e.g. strength of infection, lighting paradigm, when the phenotype was measured. Any of these could have explained the discrepancy.

Although Imd is one of the canonical immune pathways in insects, over-expression of the Imd pathway can also lead to apoptosis[21,22]. It cannot be excluded that our results could be caused by a side effect: apoptosis of the fat body by the Imd pathway rather than its main effect of immune response. This will be examined in future work.

We have shown that immune response decreases sleep and memory in *Drosophila melanogaster*. A possible link between all three systems is intriguing. One of the main hypotheses on sleep function is that sleep periods are favourable for brain plasticity and in the adult brain for learning and memory[23]. Like humans, flies with a fragmented sleep show impaired learning compared with flies with consolidated sleep[24]. Flies also exhibit decreases in learning after 6 or 12 hours of sleep deprivation[25]. We propose sleep as an intermediate between immunity and memory. We hypothesise that it is not the activation of the immune system *per se* that affects memory in flies, but rather that immune stimulation reduces the length and quality of sleep that in turn, reduces memory ability. However, with our current data, we cannot exclude that in flies the level of immune activation has a direct effect on memory and this will be the basis of future work.

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278 Figure Legends
279
280 Figure 1. Memory score for each genotype. Memory score is the proportion of flies
281 that choose the odour they were not trained against. The white boxes represent the
282 mean memory score for the RU486- flies. The grey boxes represent the RU486+ flies.
283 The grey dots are the individual data points.
284
285
286 Figure 2. Sleepbins for each genotype. The black points represent the means of
287 sleepbins for the RU486- flies. The grey points represent the RU486+ flies. The
288 shaded times are night (lights off).
289

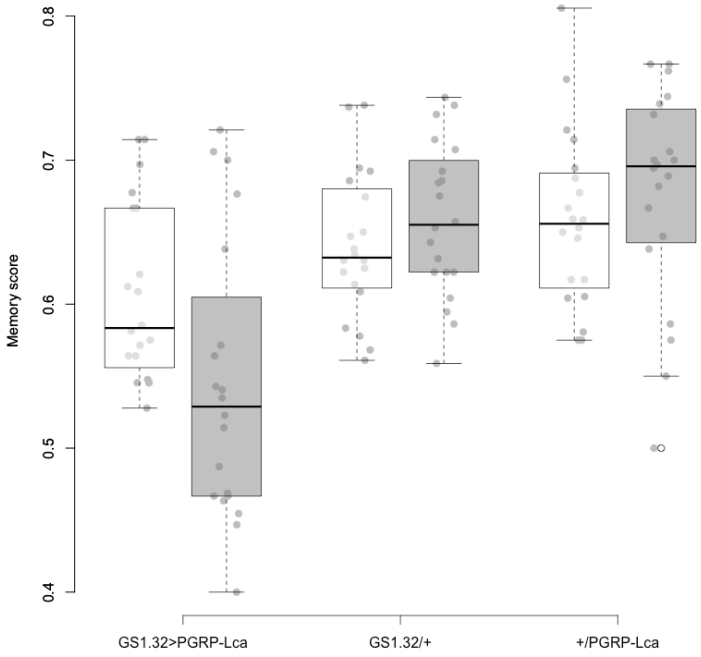

Figure 1.

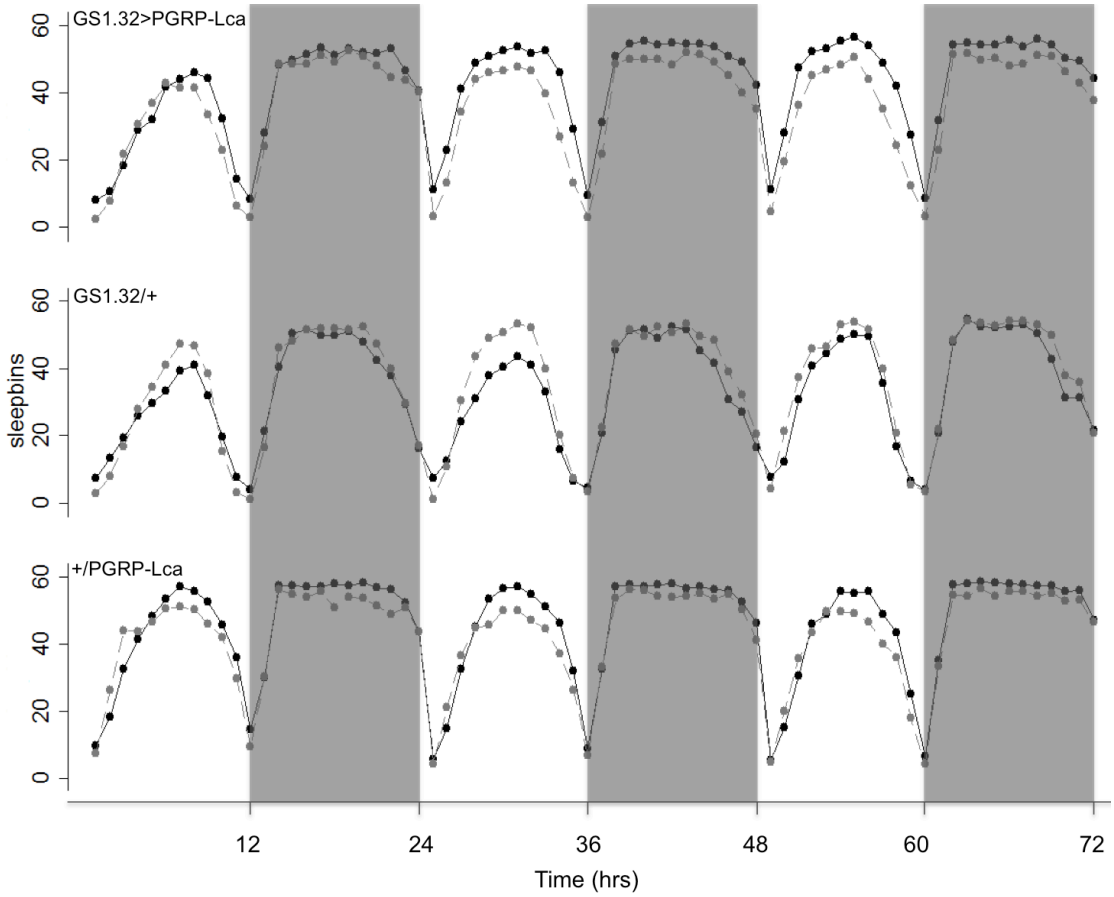

Figure 2