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

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Abstract
Psychoneuroimmunology studies the increasing number of connections between neurobiology, immunology and behaviour. 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. 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, immunology and behaviour (Ader et al. 1991). These neural-immune interactions have also been found in invertebrates (Demas et al. 2011). For example, immune response negatively affects learning and memory in bees (Mallon et al. 2003; Riddell & Mallon 2006; Gegear et al. 2006; Iqbal & Mueller 2007; Alghamdi et al. 2008). A tractable invertebrate model of these immune-neural links would provide a stimulus to this field (Aubert 2007). The fruit fly, Drosophila melanogaster, has been tremendously helpful to the analysis of associative learning (Kim et al. 2007) and immunity (Lemaitre & Hoffmann 2007). 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 (Siegel 2003). 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 (Hendricks et al. 2000; Shaw et al. 2000). Such intervals are associated with reversible increases in arousal threshold, which can be further augmented following sleep deprivation (Huber et al. 2004), are associated with changes in brain electrical activity (Nitz et al. 2002; Alphen et al. 2013), and are reduced by several drugs like caffeine and modafinil and are increased by antihistamines (Hendricks et al. 2000; Shaw et al. 2000). As in mammals, sleep deprivation leads to a rebound in quantity of sleep (Shaw et al. 2000).

Infections increase sleep in humans, most likely through induction of proinflammatory cytokines (Bryant et al. 2004). Fruit flies infected with gram-negative bacteria also
show increased sleep (Kuo et al. 2010). On the contrary, Shirasu-Hiza infected flies
with gram-positive bacteria and observed that they slept less (Shirasu-Hiza et al. 2007).
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 (Cirelli et
al. 2005; Williams et al. 2007).

Here, we activated the immune system non-pathogenically (Moret & Schmid-Hempel
2000; Mallon et al. 2003; Riddell & Mallon 2006; Alghamdi et al. 2008; Richard et al.
2008). This has the advantage that it separates the effect of the immune response from
any direct effect of the pathogen, for example, parasite manipulation of the host
(Adamo & Webster 2013). We used Geneswitch (Osterwalder et al. 2001) 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 (Gottar et al.
2002). Geneswitch is activated in the presence of the steroid RU486. We used an
aversive classical conditioning paradigm to measure memory abilities of flies (Mery &
Kawecki 2005). Sleep was measured using the Drosophila Activity Monitoring System
2 (DAMS2, Trikinetics, Waltham, MA).
Methods and Materials

The Geneswitch line $w^{1118}\{w^{mW}\text{hs}=\text{Switch1}\}^{bun}\text{Switch1.32}$ (hereafter referred to as GS1.32) drives expression of RU486-activated GAL4 in adult fat bodies (Gottar et al. 2002) (http://flystocks.bio.indiana.edu). The three genotypes used were GS1.32>PGRP-Lca ($w^{1118};\text{GS1.32}/+; UAS-PGRP-Lca/+), and the control genotypes GS1.32/+(w^{1118}, GS1.32/++; +/+), and +/PGRP-Lca (+/+; UAS-PGRP-Lca/+).

Flies were maintained in vials containing agar, sugar, and Brewer’s yeast media in a 12 h: 12 h light: dark cycle at 25°C. 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 as they sleep for over twice as long as females (Isaac et al. 2010).

Geneswitch

In the Geneswitch system, the DNA binding domain of the GAL4 protein is fused to the activation moiety of p65 through a mutant progesterone receptor ligand binding domain. Thus, Geneswitch is a chimeric ligand-stimulated activator of transcription. In the absence of ligand, the Geneswitch is in the “off” state. In the presence of the antiprogestin RU486 the Geneswitch molecule changes to an active conformation, in which it binds, as a dimer, to UAS sequences and activates transcription of downstream genes. In flies, Geneswitch mediated expression can be detectable 3–5 hr after feeding on RU486, reaching maximal levels 21–48 hr later (Roman et al. 2001; Osterwalder et al. 2001).
20 ml of RU486 (Sigma Aldrich) 10mM stock solution (0.13 g of RU486 in 32 ml of 80% ethanol) was mixed with 980 ml molten 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. This memory assay was described previously (Mery & Kawecki 2005). Conditioning consisted of 5 training sessions separated by 20min intervals. In each training session flies were first exposed for 30s to one odorant simultaneously with mechanical shock delivered every 5s. This period was followed by a 60s rest period (no odour and no shock). Then, for 30s another odorant was delivered, without shock. Flies were either conditioned against 3-octanol or 4-methylcyclohexanol (both 0.6ml/l of paraffin).

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

Fly locomotor activity was monitored by the *Drosophila* Activity Monitoring System 2 (DAMS2, Trikinetics, Waltham, MA), at 25°C, continuously for seventy-two hours under a 12:12 light:dark cycle. Output from DAMS2 was the number of times a fly crossed an infrared beam in a given 1 min period (bin). A sleep episode (bout) was defined as 5 or more consecutive bins of immobility. 384 flies were tested, divided between genotype and RU486 (presence/absence).

Data analysis for sleep assay

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

Flies sleep differently during the day and night (Ishimoto *et al.* 2012). Therefore for each dependent sleep variable, two ANOVAs one for day and one for night was run. 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, the genotypes responded differently to the treatments. To discover which genotypes were significantly different two further ANOVAs were performed, one for genotypes GS1.32>PGRP-Lca vs GS1.32/+ and one for genotypes GS1.32>PGRP-Lca vs +/- PGRP-Lca. If the interaction terms in both these ANOVAs are significant GS1.32>PGRP-Lca (the immune stimulated genotype) responses differently to the
control genotypes. Using a Bonferroni correction the significance level $\alpha$ was reduced to 0.0083 (0.05/6). All analysis was carried out using STATA12.

159 Zone of inhibition assay

160 Our treatment line had previously been shown to upregulate the immune response (Gottar et al. 2002). However we used the zone of inhibition assay to confirm increased immune response in our treated flies. 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 (*Arthrobacter 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 pipetted 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-
Lca vs GS1.32/+: $F_{1,4607} = 16.38, p < 0.00001$, GS1.32>PGRP-Lca vs +/- PGRP-Lca:

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

Immune stimulated adult flies 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. 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 immune-stimulated 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 (Shirasu-Hiza et al. 2007). However Kuo (Kuo et al. 2010) 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 discrepancies between these two previous results are difficult to explain as the experiments differed in numerous other methodical aspects, e.g. strength of infection, lighting paradigm, when the phenotype was measured.

Although Imd is one of the canonical immune pathways in insects, over-expression of the Imd pathway can also lead to apoptosis (Georgel et al. 2001; Leulier et al. 2003). 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*. We propose a possible link between all three systems as an interesting
area for future research. 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 (Maquet 2001). Like humans, flies with a fragmented sleep show impaired
learning compared with flies with consolidated sleep (Seugnet *et al.* 2008). Flies also
exhibit decreases in learning after 6 or 12 hours of sleep deprivation (Seugnet *et al.*
2006 p. 200). 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.

Our results establishes *Drosophila* as a model for immune-neural interactions. As well
as the potential use as a model for mammalian neural-immune links, this work has
direct impact on recent concern for insect foragers and the role of multiple stressors in
their decline (Gill *et al.* 2012).
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References


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Figure Legends

Figure 1. Memory score for each genotype. Memory score is the proportion of flies that choose the odour they were not trained against. The white boxes represent the mean memory score for the RU486- flies. The grey boxes represent the RU486+ flies. The grey dots are the individual data points.

Figure 2. Sleepbins for each genotype. The black points represent the means of sleepbins for the RU486- flies. The grey points represent the RU486+ flies. The shaded times are night (lights off).
Figure 1
Figure 2