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The visual orientation memory of *Drosophila* requires Foraging (PKG) upstream of Ignorant (RSK2) in ring neurons of the central complex

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Orientation and navigation in a complex environment requires path planning and recall to exert goal-driven behavior. Walking *Drosophila* flies possess a visual orientation memory for attractive targets which is localized in the central complex of the adult brain. Here we show that this type of working memory requires the cGMP-dependent protein kinase encoded by the *foraging* gene in just one type of ellipsoid-body ring neurons. Moreover, genetic and epistatic interaction studies provide evidence that Foraging functions upstream of the Ignorant Ribosomal-S6 Kinase 2, thus revealing a novel neuronal signaling pathway necessary for this type of memory in *Drosophila*.

To analyze a visual orientation memory, we have developed the so-called detour paradigm in which flies walk on a circular, water-surrounded platform within a computerized cylindrical LED screen (Fig. 1; Neuser et al. 2008). In this assay, flies that are confronted with two opposing, inaccessible, and vertical dark stripes on the brightly illuminated cylinder will walk back and forth between the stripes for a considerable amount of time. When the fly is heading toward one stripe (Fig. 1A), thereby crossing the virtual midline of the platform, the stripes disappear and simultaneously a distracting stripe shows up laterally to the fly. The new target lures the fly out of its original pathway and it tries to approach the distracter (Fig. 1B). When the distracting stripe also disappears, the fly remembers the position of the original stripe and resumes walking into the direction of its original target (Fig. 1C).

Throughout this study 3- to 5-d-old flies, with their wings clipped under cold anesthesia 1 d prior to testing, were tested in the detour paradigm as described in Neuser et al. (2008). Ten approaches per fly were recorded and the percentage of choices toward the initial target was calculated for each fly (% positive choices). Wild-type Canton-S (CS) flies recall the position of the initial target with a median frequency of 80% (Fig. 1D). Normality was tested with the Shapiro-Wilk Test. We used the Kruskal–Wallis Analysis of Variance Test for multiple comparisons and the Wilcoxon Matched Pairs Test for dependent samples in a one-by-one analysis. The nonparametric comparison against the random value was carried out with the one-sample Sign Test. The suitable test for a parametric comparison was the single t-test. Statistical analyses were performed with Statistica 7.0 and SPSS 20. See Supplemental Table S1 for all statistical calculations and numbers of flies tested.

To investigate the role of FOR in visual orientation memory, we used the dominant forR allele, the recessive hypomorph forS.
and another recessive allele (for<sup>189Y</sup>) that was isolated in a P[GAL4]-enhancer trap screen (Osborne et al. 1997; Wang et al. 2008). Homozygous Rover flies (for<sup>R</sup>/for<sup>R</sup>) possess a wild-type orientation memory when compared to Canton-S controls (CS) (Fig. 1D). In contrast, homozygous sitter flies (for<sup>s</sup>/for<sup>s</sup>) display no memory for the initial target, because their positive choices were not different from chance level. The chance level differs from 50% because a fly that has turned to the right is more prone to make a following left turn. Therefore, a preference index of 58% represents random behavior, i.e., loss of orientation memory (Neuser et al. 2008). The analysis of sitter mutants transheterozygous for the hypomorphic for<sup>s</sup> and for<sup>189Y</sup> alleles revealed a considerable reduction of the orientation memory in the detour paradigm when compared to for<sup>s</sup>/for<sup>s</sup> flies (Fig. 1D). However, transheterozygous for<sup>s</sup>/for<sup>189Y</sup> flies still possess a residual memory because their positive reactions for the initial target were significantly different from the 58% chance level. A similar result was obtained with flies transheterozygous for for<sup>189Y</sup> and a deficiency (Dif(2)ED243) (Tweedie et al. 2009) that specifically deletes a large part of the for gene locus. Moreover, heterozygous for<sup>s</sup>/for<sup>189Y</sup> flies displayed wild-type behavior, corroborating the recessive nature of this for allele (Fig. 1D). Loss of orientation memory was also observed by inducing RNA interference against the for transcription unit specifically in the ellipsoid-body ring neurons, further supporting the finding that FOR is required for visual orientation memory (see Supplemental Fig. S2).

Next we asked whether FOR function is needed in the same subtypes of ring neurons of the ellipsoid body as IGN. To address this, we performed tissue-specific cDNA rescue experiments in a for<sup>s</sup>/for<sup>s</sup> mutant background using the UAS/GAL4-expression system (Brand and Perrimon 1993). Expression of FOR using the GAL4 driver line c232, which is specific for ring neuron types R3 and R4d (Renn et al. 1999), also restored the for<sup>s</sup> mutant deficits to wild-type levels (Fig. 2A,D); this GAL4 line has been shown to rescue the orientation memory loss of the ign mutant (Neuser et al. 2008). To differentiate between the two sets of ring neurons targeted by the c232-GAL4 line, we made use of the 189Y-GAL4 line that expresses only in R3 ring neurons (Fig. 2C; Renn et al. 1999). Although this driver line represents a hypomorphic allele of the for gene (Fig. 1D; Osborne et al. 1997; Wang et al. 2008), the GAL4 expression pattern seems to only partially reflect the endogenous FOR pattern (Belay et al. 2007; Supplemental Fig. S1). A recent analysis of the transposon insertion site has shown that a P-element integrated into the lilli gene locus (Wang et al. 2008) at position 23C, whereas for localizes to 24A. Therefore, the GAL4 expression pattern might reflect the expression pattern of lilli. Nevertheless, 189Y-driven GAL4 and endogenous FOR are expressed in a similar pattern, including the ring neurons of the ellipsoid body and the α, β, and γ lobes of the mushroom bodies (Belay et al. 2007; Mery et al. 2007). In addition, 189Y induces expression of GAL4 in neurosecretory cells in the pars intercerebrals and in GABAergic interneurons of the antennal lobes (Lebestky et al. 2009; for a larger image of the section presented in Fig. 2C and a detailed expression analysis of the adult brain, see Supplemental Fig. S1). Indeed, expression of FOR using 189Y-GAL4 rescued the memory deficits of for<sup>s</sup>/for<sup>189Y</sup> heterozygous mutants to wild-type levels, thus establishing that FOR is only necessary in the R3 neurons to restore visual orientation memory. As a control we used driver line 201Y-GAL4, which induces FOR in the same compartments (a-/b-/γ-lobes) (Aso et al. 2009) of the mushroom body as 189Y-GAL4. This did not restore visual orientation memory, verifying that expression in the mushroom body did not play a role in the rescue with 189Y-GAL4. Next we asked whether the other types of ring neurons are also able to restore the for<sup>s</sup> mutant orientation memory phenotype. However, using three additional driver lines to cover all other ring neurons (Renn et al. 1999) did not rescue the for phenotype (Fig. 2A). Therefore, FOR is only necessary and required in R3 neurons for the visual orientation memory.

The for gene is also expressed during development (Belay et al. 2007) and lethal alleles have been reported (Tweedie et al. 2009). To exclude the possibility that the reduced FOR function during development of the adult nervous system is negatively influencing adult orientation memory in for<sup>s</sup> mutant flies, we used an inducible expression system to confine FOR expression to the adult stage. The combination of the UAS-GAL4 system with the ubiquitously expressed, temperature-sensitive GAL4 repressor GAL80<sup>Tub</sup>-[>GAL80<sup>Tub</sup>] (McGuire et al. 2003) provides temporal control of FOR expression that can be induced by elevating the temperature. We therefore reared 189Y-GAL4-driven rescue flies at the restricted temperature (18°C) and tested them for their orientation memory and, as expected, these flies show the memory defect (Fig. 2B). The flies were then shifted to 30°C overnight to induce additional FOR expression, tested again, and compared by a paired statistical analysis (Supplemental Table S1). This treatment rescued the memory deficits in for mutants, showing that additional expression of FOR in the adult ring neurons is sufficient to restore visual orientation memory (Fig. 2B).
As mentioned above, like FOR, the IGN kinase is also required in the ring neurons of the adult brain for orientation memory (Neuser et al. 2008). To ascertain whether both genes act in the same genetic pathway, we conducted a genetic interaction study by reducing the gene copy number of ign in a hypomorphic for mutant background. Although transheterozygous for for flies over a deficiency, and homozygous for for display no orientation memory (Figs. 1D, 2A), transheterozygous for for animals have an intermediate phenotype that is, however, still significantly different from wild-type and random levels (Fig. 1D). We therefore used this combination for genetic interaction studies because it allows us to detect suppressing as well as enhancing effects. Comparing for for flies with flies that have in addition only one copy of the ign gene (ign+/+; for for) (Fig. 3A) revealed a stronger memory deficit that was reduced to random levels. In contrast, removing one wild-type copy of the ign gene in for for heterozygous animals had no effect on the orientation memory (Fig. 3A). The genetic interaction of ign with the recessive for alleles suggests that both kinases act in the same signaling pathway and that both promote memory formation.

Having established a connection between the FOR and IGN kinases, we then aimed to elucidate the hierarchy of these two interacting proteins. We therefore investigated whether overexpression of IGN in the R3 ring neurons of the ellipsoid body could rescue the for phenotype. Indeed, 189Y-GAL4 driven overexpression of IGN rescued the memory deficits of transheterozygous for mutants completely (Fig. 3B). This result suggests that IGN functions downstream from FOR in R3 ring neurons. To confirm this finding, we performed the reverse experiment by overexpressing FOR in an ign mutant background. Indeed, neither c232-GAL4 nor 189Y-GAL4 driven FOR overexpression was able to mitigate or rescue the memory deficits in ign mutants, thus further supporting our hypothesis that FOR acts upstream of IGN (Fig. 3C).

Interestingly, this interaction pathway does not seem to play a role in another type of short-term memory that is required for orientation in the so-called heatbox paradigm. A gain-of-function allele of the ign gene negatively affects the conditional place preference in the heatbox (Putz et al. 2004), whereas Rover and sitter flies do not differ in their memory performance (Gioia and Zars 2009).

FOR signaling has previously been implicated in different types of memories; however, in contrast to the working memory in the detour paradigm, these memories require a longer time frame to be established (e.g., Hofmann et al. 2006). In mammals, nitric oxide, the initiating molecule of the cGMP/PKG-pathway, is thought to act as a retrograde messenger during the induction of long-term potentiation (LTP) (Hawkins et al. 1998; Taqatqeh et al. 2009). Zhuo et al. (1994) reported a LTP enhancement after adding PKG activators and a long-term depression after the addition of PKG inhibitors. Mice carrying a knock-out for the PKG gene show reduced ability of motor learning due to a loss of synaptic plasticity in the
cerebellum (Feil et al. 2003). Furthermore, mice lacking PKG in the amygdala exhibit an impairment in fear conditioning (Paul et al. 2008) and cGMP/PKG signaling in the hippocampus is required for novel object recognition (Feil et al. 2009). In insects, FOR is involved in different types of food searching behavior and associative memories in which establishing the learning traces takes at least seconds (Réaume and Sokolowski 2009). In contrast, the orientation memory observed in the detour paradigm represents a form of working memory which has to be updated continuously in fractions of seconds. Whereas the phosphorylation and activation of FOR and IGN might be the mechanism by which these kinases affect longer-lasting memories, we think it is unlikely that this mechanism is involved in the constantly and rapidly changing orientation memory. Both kinases would have to be activated or inactivated in an online fashion during every turn of the fly. On the other hand, RSK2 has been implicated in multiple cellular processes and transcriptional control (Romeo et al. 2012). We therefore like to speculate that the biochemical pathway both kinases work in is necessary to endow the ring neurons with the capacity to efficiently change signaling rapidly to encode orientation. For instance, ring neurons might need a higher density of synaptic release sites and/or dendritic neurotransmitter receptors to exert their specific function.

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