Cellular site and molecular mode of synapsin action in associative learning

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Synapsin is an evolutionarily conserved, presynaptic vesicular phosphoprotein. Here, we ask where and how synapsin functions in associative behavioral plasticity. Upon loss or reduction of synapsin in a deletion mutant or via RNAi, respectively, Drosophila larvae are impaired in odor-sugar associative learning. Acute global expression of synapsin and local expression in only the mushroom body, a third-order “cortical” brain region, fully restores associative ability in the mutant. No rescue is found by synapsin expression in mushroom body input neurons or by expression excluding the mushroom bodies. On the molecular level, we find that a transgenically expressed synapsin with dysfunctional PKA-consensus sites cannot rescue the defect of the mutant in associative function, thus assigning synapsin as a behaviorally relevant effector of the AC-cAMP-PKA cascade. We therefore suggest that synapsin acts in associative memory trace formation in the mushroom bodies, as a downstream element of AC-cAMP-PKA signaling. These analyses provide a comprehensive chain of explanation from the molecular level to an associative behavioral change.

[Supplemental material is available for this article.]
Figure 1. The chemosensory pathways of Drosophila larva and the requirement of synapsin for associative function. (A) SEM image of the larval head (courtesy of M. Koblofsky). (B) Cephalic chemosensory pathways in the larva (modified from Stocker 2008, with permission from Landes Bioscience and Springer Science+Business Media © 2008). (C) The odor–sugar associative learning paradigm. Circles represent petridishes containing a sugar reward (orange, +) or only pure agarose (white). Animals are trained either AM+/OCT or OCT+/AM and then tested for choice between AM vs. OCT (for half of the cases, the sequence of training trials is reversed: OCT/AM+ and AM/OCT+). (D) Dorsal view of a Drosophila larval brain with the major brain regions reconstructed. The inset shows a magnified view of MB, PN, and AL (see also Supplemental Movie S1). (E–K) Associative impairment of syn97 mutants is interpretable without reference to white function. (E–I) Anti-synapsin (white) and anti-F-actin (orange) immunoreactivity of brains of the indicated genotypes; the Western blot shows the expected bands at 74 and 143 kDa. (K) In syn97 and w1118; syn97 mutants, associative function is reduced by half; the w1118 mutation has no effect. Box plots marked with different letters indicate significant differences in associative ability (P < 0.05/4). (L,M) Associative function is impaired upon knock-down of synapsin by RNAi. (L) Western blot from brains of larval Drosophila of the indicated genotypes. Synapsin expression is reduced in the brain-wide KNOCK-DOWN larvae. (M) Associative function is impaired in the brain-wide KNOCK-DOWN strain. Box plots marked with different letters indicate significant differences in associative ability (P < 0.05/2). MH, mouth hook; dorsal, terminal, ventral organ (DO, TO, VO) and their ganglia (DOG, TOG, VOG); AL, antennal lobe; PN, projection neurons; MB, mushroom body; P, peduncle of the MB; KC, Kenyon cells comprising the MB; LH, lateral horn; antennal, labral, maxillary, labial nerve (AN, LN, MN, LBN); dorsal, ventral, posterior pharyngeal sense organ (DPS, VPS, PPS); LN, local interneurons; PN, projection neurons; iACT, inner antennocerebral tract; SOG, subesophageal ganglion; the orange arrowheads indicate aminergic reinforcement neurons toward the mushroom bodies; the pharynx is shown stippled; VNC, ventral nerve cord. Scale bars: 50 µm.
formance of transgenic larvae carrying w1118 in DRIVER control larvae (Fig. 1M; MW: 19.03; df = 3; N = 9, 7, 7, 10). Specifically, the brain-wide RESCUE larvae perform better than EFFECTOR control larvae (Fig. 2E; MW: P < 0.05/3, U = 4.5). Importantly, associative ability is restored fully in the brain-wide RESCUE larvae, i.e., they do as well as wild-type CS larvae (Fig. 2E; MW: P > 0.05/3; U = 28). Thus, a brain-wide rescue of synapsin is sufficient to fully restore the syn97 mutant associative defect.

Induced rescue

To see whether the defect in associative function upon lack of synapsin is indeed due to an acute requirement of synapsin, we induce expression acutely before the behavioral experiment.

Results

Associative defect of syn97 mutants phenocopied by RNAi

We have shown (Michels et al. 2005) that larvae lacking synapsin (syn97) show a 50% reduction in an odor–sugar associative learning paradigm but show intact ability to (1) taste, (2) smell, and (3) move to and from the test arena; also, susceptibility to (4) the stress of handling, (5) olfactory adaptation, and (6) changes of motivation as caused by the experimental regimen are unaltered. Here, we first confirm the lack of synapsin (Fig. 1F,H,I) and the associative defect of syn97 larvae: Wild-type CS show about twice as high associative performance indices compared to syn97 mutants (Fig. 1K; MW: P < 0.05; U = 106; N = 28, 16). The same defect is uncovered comparing between w1118 and w118, syn97 larvae (Fig. 1K; MW: P < 0.05; U = 44; N = 16, 13). This shows that the defect of syn97 larvae in odor–sugar associative learning—and thus performance of transgenic larvae carrying w118 as marker—can be interpreted without reference to white function.

Next, using RNAi, we find that synapsin levels are indeed reduced (Fig. 1L), and concomitantly associative performance indices in the KNOCK-DOWN larvae are about 50% lower than in EFFECTOR control larvae (Fig. 1M; MW: P < 0.05/2, U = 408), and in DRIVER control larvae (Fig. 1M; MW: P < 0.05/2, U = 441) (KW: P < 0.05; H = 8.00; df = 2; N = 36, 37, 34). Thus, a reduction of synapsin by means of RNAi causes an associative impairment which phenocopies the defect in the syn97 null mutant.

Brain-wide rescue

In brain-wide RESCUE larvae, synapsin expression is restored throughout the brain (Fig. 2B; Supplemental Fig. S1B–D; Supplemental Movie S2). Comparing performance scores between genotypes shows a difference in associative ability (Fig. 2E; KW: P < 0.05/H = 19.03; df = 3; N = 9, 7, 7, 10). Specifically, the brain-wide RESCUE larvae perform better than EFFECTOR control larvae (Fig. 2E; MW: P < 0.05/3, U = 0) and DRIVER control larvae (Fig. 2E; MW: P < 0.05/3, U = 4.5). Importantly, associative ability is restored fully in the brain-wide RESCUE larvae, i.e., they do as well as wild-type CS larvae (Fig. 2E; MW: P > 0.05/3; U = 28). Thus, a brain-wide rescue of synapsin is sufficient to fully restore the syn97 mutant associative defect.

Induced rescue

To see whether the defect in the associative function upon lack of synapsin is indeed due to an acute requirement of synapsin, we induce expression acutely before the behavioral experiment.

Figure 2. Brain-wide (A–E) and induced (F–L) rescue. (A–E) Constitutive and (F–L) induced expression of synapsin. (A–D, F–I) Anti-synapsin (white) and anti-F-actin (orange) immunoreactivity of brains of the indicated genotypes. (A–D) Synapsin expression is detected in wild-type CS and in the brain-wide RESCUE strain. (E) Associative function is fully rescued in the brain-wide RESCUE strain. (F–I) With heat shock, synapsin expression is seen in wild-type CS and induced brain-wide RESCUE larvae; (F′–I′) without heat shock, synapsin staining is detected only in the wild-type CS strain. (K) Associative function is fully rescued by induced synapsin expression; without heat shock (L), no rescue is observed. Scale bars: 50 μm. All other details as in the legend of Figure 1.
Upon heat shock (HS) to induce synapsin expression, both wild-type CS and induced brain-wide RESCUE larvae show synapsin expression throughout the brain (Fig. 2F,G). However, the genetic controls do not show synapsin expression (Fig. 2H,I). When no HS is applied, synapsin is found only in the wild-type CS, but in neither of the other genotypes (Fig. 2F–I). With regard to associative ability, the four genotypes differ after HS (Fig. 2K; KW: \( P = 0.05; \) \( H = 18.37; \) \( df = 3; N = 8, 10, 8, 12 \)). Importantly, induced brain-wide RESCUE larvae show the same associative performance indices as wild-type CS larvae (Fig. 2K; MW: \( P = 0.79; \) \( U = 37 \)). Also, upon HS the induced brain-wide RESCUE larvae perform significantly better than EFFECTOR control (Fig. 2K; MW: \( P < 0.05/3; \) \( U = 11 \)) and than brain-wide DRIVER control larvae (Fig. 2K; MW: \( P < 0.05/3; \) \( U = 11 \)). When no HS is given, associative performance scores expectedly also show a significant difference between the four genotypes (Fig. 2L; KW: \( P < 0.05; \) \( H = 12.95; \) \( df = 3; N = 9, 12, 9, 8 \)); however, without HS the induced brain-wide RESCUE larvae show significantly lower scores than wild-type CS (Fig. 2L; MW: \( P < 0.05/3; \) \( U = 16 \)) and do not differ from EFFECTOR control (Fig. 2L; MW: \( P > 0.05/3; \) \( U = 47 \)) and brain-wide DRIVER control larvae (Fig. 2L; MW: \( P > 0.05/3; \) \( U = 44 \)). Therefore, associative function is restored fully when synapsin expression is acutely induced, suggesting an acute function of synapsin in associative processing.

Local rescue at mushroom bodies

We next ask whether synapsin expression in only the mushroom bodies will restore the defect of the \( \text{syn}^{97} \) mutants in associative function. Associative performance scores differ between wild-type CS, mushroom-body RESCUE strain, DRIVER control, and EFFECTOR control (Fig. 3E; KW: \( P < 0.05; \) \( H = 21.39; \) \( df = 3; N = 10, 11, 10, 11 \)). Mushroom-body RESCUE larvae show associative scores indistinguishable from wild-type CS (Fig. 3E; MW: \( P = 0.62; \) \( U = 48 \)), but better than mushroom-body DRIVER control (Fig. 3E; MW: \( P < 0.05/3; \) \( U = 11 \)) and EFFECTOR control larvae (Fig. 3E; MW: \( P < 0.05/3; \) \( U = 18 \)). We therefore conclude that synapsin expression in the mushroom body, as covered by the \( \text{mb}247\text{–}\text{Gal4} \) driver (Fig. 3B, B′), is sufficient to fully rescue the \( \text{syn}^{97} \) mutant defect in an odor–sugar associative learning paradigm.

In terms of expression pattern, \( \text{mb}247\text{–}\text{Gal4} \) leads to synapsin expression in all basic compartments of the larval mushroom body, i.e., calyx, peduncle, and lobes (Fig. 3B, B′; Supplemental Fig. S1E,F; Supplemental Movie S3), covering ~300 larval mushroom body neurons.

We next ask whether a rescue of associative function can also be found if drivers are used that cover fewer mushroom body neurons. Crossing the \( \text{DS}2\text{H}\text{–}\text{Gal4} \) driver to a UAS–GFP effector strain, we observe that expression is found in indeed few mushroom body neurons (seven mushroom body neurons per hemisphere; Supplemental Fig. S1G,H). Notably, although only so few mushroom body neurons are covered, GFP expression reveals the basic compartments of the larval mushroom bodies; in particular, the mushroom body input regions (the calyx) seem to be covered fairly well (Supplemental Fig. S1G,H; Supplemental Movie S4). The same holds true for synapsin expression if the \( \text{DS}2\text{H}\text{–}\text{Gal4} \) driver is recombined into the \( \text{syn}^{97} \)-mutant background and crossed to our rescue effector strain (Fig. 3G, G′).

Using the \( \text{DS}2\text{H}\text{–}\text{Gal4} \) driver, we find that wild-type CS, the mushroom-body–subset RESCUE strain, and its genetic controls differ in associative performance indices (Fig. 3K; KW: \( P < 0.05; \) \( H = 13.85; \) \( df = 3; N = 12, 10, 12, 12 \)). Mushroom-body–subset RESCUE larvae do just as well as wild-type CS (Fig. 3K; MW: \( P = 0.55; \) \( U = 51 \)), whereas they perform better than either mushroom-body–subset DRIVER control (Fig. 3K; MW: \( P < 0.05/3; \) \( U = 18 \)) or EFFECTOR control larvae (Fig. 3K; MW: \( P < 0.05/3; \) \( U = 21.0 \)). This suggests that synapsin expression in only a handful of mushroom body neurons, defined by expression from the \( \text{DS}2\text{H}\text{–}\text{Gal4} \) driver, can be sufficient to rescue the \( \text{syn}^{97} \)-mutant defect in associative function.

Figure 3. Local rescue at the mushroom bodies. (A–D, F–I) Anti-synapsin (white) and anti-F-actin (orange) immunoreactivity of brains of the indicated genotypes; in B′ and G′, a magnified view of the mushroom body is presented. (E) Associative function is fully rescued in the mushroom-body RESCUE strain. (F–K) Local rescue in a small subset of mushroom body neurons by using a mushroom-body subset driver (\( \text{DS}2\text{H}\text{–}\text{Gal4} \)). Associative function is fully rescued in the mushroom-body subset RESCUE strain. Calyx (Cx), peduncle (P), vertical lobe (VL), medial lobe (ML). Scale bars: 50 \( \mu \text{m} \) in A–D and F–I, 25 \( \mu \text{m} \) in B′ and G′. All other details as in the legend of Figure 1.
No rescue at projection neurons

Given that in bees (reviewed in Menzel 2001) and adult flies (Thum et al. 2007) the projection neurons have been suggested as an additional site of an odor–sugar memory trace, we next test whether associative function is restored in projection–neuron RESCUE larvae compared to their genetic controls and wild-type CS. Associative performance indices between these genotypes are different (Fig. 4E; KW: $P < 0.05$; $H = 19.15$; df = 3; $N = 10, 10, 10, 10$). Importantly, however, projection–neuron RESCUE larvae show scores significantly smaller than wild-type CS (Fig. 4E; MW: $P < 0.05$/3; $U = 9$) and indistinguishable from either genetic control (Fig. 4E; projection–neuron RESCUE vs. projection–neuron DRIVER control: MW: $P > 0.05$/3; $U = 43.5$; projection–neuron RESCUE vs. EFFECTOR control: MW: $P > 0.05$/3; $U = 46$).

However, as is the case for any lack of rescue, the insertion of the driver construct may produce haploinsufficiency in the gene(s) neighboring it, and this haploinsufficiency may lead to a learning defect masking an actually successful rescue.

![Figure 4](https://learnmem.cshlp.org)
Therefore, we compare larvae heterozygous for the used projection–neuron driver construct (GH146–Gal4) to wild-type CS and w1118 mutant larvae. Associative performance indices of these three genotypes are indistinguishable (Fig. 4F; KW: P > 0.05; H = 0.04; df = 2; CS: N = 10, 10, 10). Thus, expression of synapsin in projection neurons, as covered by GH146–Gal4, is not sufficient for rescuing the syn97 mutant defect in a larval odor–sugar associative learning paradigm. This lack of rescue cannot be attributed to a haploinsufficiency caused by the insertion of the GH146–Gal4 construct.

Regarding the expression pattern of synapsin supported by GH146–Gal4, we note that consistent with what has been reported previously (Marin et al. 2005; Masuda-Nakagawa et al. 2005; Ramakers et al. 2005), a substantial fraction of the projection neurons (at least 13–16 of the total of about 21) are expressing synapsin. Correspondingly, we observe expression throughout the input and output regions of the projection neurons (antennal lobe, mushroom body calyx, lateral horn: Fig. 4B,B′). Obviously, however, expression is not restricted to the projection neurons (see also Heimbeck et al. 2001; Thum et al. 2007): strong expression is seen in the optic lobe Anlagen, a site where in the wild-type CS strain no synapsin is expressed (asterisk [∗] in Fig. 4B). As synapse formation in the lamina emerges at the earliest in the midpupal period, this expression likely is without consequence in our paradigm. Finally, when assayed via GFP expression, we uncover expression in a mushroom body-extrinsic neuron (Supplemental Fig. S11–L; Supplemental Movie S5; see also Heimbeck et al. 2001). Possibly, such expression remains unrecognized in terms of synapsin immunoreactivity. Given that all these behavioral and histological conclusions are confirmed using Np225–Gal4 as another projection–neuron RESCUE strain (Fig. 4G–M; Supplemental Fig. S1M–O; Supplemental Movie S6), a rescue of the associative defect in the syn97 mutant does not appear to be possible in the projection neurons.

**Scrutinizing the lack of rescue at projection neurons**

Of all available fly strains, GH146–Gal4 and Np225–Gal4 express broadest and strongest in the projection neurons. Still, about one-third of the projection neurons of the larva are not covered. Therefore, it is possible that within the Gal4 expression pattern, activity evoked by both odors is the same, whereas those projection neurons that allow making a difference between both odors could be spared from Gal4 expression. We therefore tested the projection neuron rescue larvae in a one-odor paradigm (Saumweber et al. 2011), such that one of the two odors is omitted. That is, larvae receive either paired or unpaired presentations of odor and reward, and then are assayed for their preference for the trained odor (Fig. 4N). In such an experiment, projection–neuron RESCUE larvae show associative performance indices significantly smaller than wild-type CS (for AM: Fig. 4O; MW: P < 0.05/3; U = 23; N = 12, 12; for OCT: Fig. 4P; MW: P < 0.05/3; U = 32, N = 13, 13) and indistinguishable from either genetic control (for AM: Fig. 4O; projection–neuron RESCUE vs. projection–neuron DRIVER control: MW: P > 0.05/3; U = 63; projection–neuron RESCUE vs. EFFECTOR control: MW: P > 0.05/3; U = 66.5; N = 12, 12, 12; for OCT: Fig. 4P; projection–neuron RESCUE vs. projection–neuron DRIVER control: MW: P > 0.05/3; U = 69; projection–neuron RESCUE vs. EFFECTOR control: MW: P > 0.05/3; U = 60; N = 13, 13, 13) (KW: for AM, Fig. 4O: P < 0.05; H = 13.5; df = 3; N = 12 for all groups; for OCT, Fig. 4P: P < 0.05; H = 12.0; df = 3; N = 13 for all groups). Thus, despite sincere efforts, there is no evidence that synapsin expression in the projection neurons, as covered by the broadest and strongest expressing driver strains available, were sufficient to restore associative function in syn97-mutants.

**No rescue without mushroom body expression**

Given that synapsin expression in the mushroom body, but not in projection neurons, is sufficient to restore the defect of the syn97 mutant in associative function, we asked whether mushroom body expression of synapsin in turn would be required. Comparing associative ability in no-mushroom body RESCUE larvae to wild-type CS and to their genetic controls (no-mushroom body DRIVER control and EFFECTOR control) reveals a significant difference (Fig. 5E; KW: P < 0.05; H = 14.40; df = 3; N = 12, 12, 12, 12). Importantly, the no-mushroom body RESCUE larvae do not show associative performance scores as high as wild-type CS (Fig. 5E; MW: P < 0.05/3; U = 24); rather, associative ability is as poor as in the genetic controls (Fig. 5E; no-mushroom body RESCUE vs. EFFECTOR control: MW: P > 0.05/3; U = 68; no-mushroom body RESCUE vs. DRIVER control: MW: P > 0.05/3; U = 69.5). Such lack of rescue cannot be attributed to a haploinsufficiency caused by the insertion of the mb247–Gal80 construct (Fig. 5F; KW: P > 0.05; H = 1.15; df = 2; N = 13, 11, 12).

A comparison of synapsin expression with repression in the mushroom bodies (by virtue of mb247–Gal80) (Fig. 5B) to synapsin expression without such repression (i.e., without mb247–Gal80) (Fig. 2B) reveals a full abolishment of expression in the mushroom bodies. Considering expression of a GFP reporter (Fig. 5G,H), however, suggests that mb247–Gal80 (1) may spare some mushroom body expression and (2) leads to a reduction of expression also outside the mushroom body (as previously noted by Ito et al. 2003). Such possible discrepancies must remain unrecognized if the expression of the actual effector is not documented. In our case, it is possible that (1) detection of GFP is more sensitive.

![Figure 5](https://www.learnmem.org/337/Learning-Memory)
than detection of synapsin; (2) the mb247-element supports different expression patterns in the mb247-Gal4 strain compared to the mb247–Gal80 strain; or that (3) Gal80 has non–cell-autonomous effects. We conclude that synapsin expression outside of the coverage of mb247–Gal80 is not sufficient to rescue the associative defect in the syn97-mutant. In turn, those neurons that are covered by mb247–Gal80 do not need to express synapsin to support associative function.

No rescue with PKA site defective synapsin
Since properly regulated AC–cAMP–PKA signalling has been shown to be necessary for olfactory short-term memory in Drosophila (see Discussion), we decided to test whether the two predicted PKA sites of the synapsin protein are required for normal learning. Therefore, we expressed a mutated synapsin protein that cannot be phosphorylated at these two predicted PKA sites because the serines of these PKA consensus sites (S-6 and S-533) were replaced by alanine (PKA-AlaAla) (for details see sketch in Fig. 6). Comparing associative ability in such SynapsinPKA-AlaAla–RESCUE larvae to wild-type CS and to their genetic controls reveals a significant difference (Fig. 6E; KW: \( P < 0.05; H = 12.24; df = 3; N = 17 \) of all groups). Importantly, the SynapsinPKA-AlaAla–RESCUE larvae do not perform as well as wild-type CS (Fig. 6E; MW: \( P < 0.05/3; U = 70 \)); rather, associative ability is as poor as in the genetic controls (Fig. 6E; SynapsinPKA-AlaAla–RESCUE vs. EFFECTOR control: \( MW: P > 0.05/3; U = 130.5; \) SynapsinPKA-AlaAla–RESCUE vs. DRIVER control: \( MW: P > 0.05/3; U = 121 \)). Such lack of rescue cannot be attributed to a haploinsufficiency caused by the insertion of the UAS–synPKA-AlaAla construct (Fig. 6F; KW: \( P > 0.05; H = 0.04; df = 2; N = 12 \) for all groups) (for a repetition of these experiments with an independent insertion of the same effector construct see Fig. 6G–M). Thus, intact PKA sites of synapsin are required to restore associative ability in the syn97-mutant.

Discussion
The associative defect in the syn97-mutant (Fig. 1K; Michel et al. 2005) can be phenocopied by an RNAi-mediated knock-down of synapsin (Fig. 2K,L). In terms of site of action, locally restoring synapsin in the mushroom bodies fully restores associative ability (Fig. 3E,K), whereas restoring synapsin in the projection neurons does not (Fig. 4E,L). If synapsin is restored in wide areas of the brain excluding the mushroom bodies, learning ability is not restored either (Fig. 5E). We therefore conclude that a synapsin-dependent memory trace is located in the mushroom bodies, and suggest that this likely is the only site where such a trace is established regarding odor–sugar short-term memory in larval Drosophila. In terms of mode of action, we find that a synapsin protein that carries dysfunctional PKA sites (Fig. 6E,L) cannot rescue the syn97-mutant learning defect. We therefore suggest that synapsin functions as a downstream element of AC–cAMP–PKA signaling in associative function.

Mode of action: synapsin as target of the AC–cAMP–PKA cascade
Arguably, the Rutabaga type I adenyl cyclase acts as a detector of the coincidence between an amnergic reinforcing signal (appetitive learning: octopamine; aversive learning: dopamine) (Schwaerzel et al. 2003; Riemensperger et al. 2005; Schroll et al. 2006) and the odor-specific activation of the mushroom body neurons (Fig. 6N). Initially, this notion had been based on mutant and biochemical analyses in Drosophila (Livingstone et al. 1984; Dудai 1985; Heisenberg et al. 1985) and physiology in Aplysia (Brunelli et al. 1976; Hawkins 1984; Yovell et al. 1992; Byrne and Kandel 1996; Abrams et al. 1998). Indeed, activation of mushroom body neurons in temporal coincidence with dopamine application increases cAMP levels in wild-type, but not AC-deficient flies (rut2080) (Tomchik and Davis 2009), and Gervasi et al. (2010) show a corresponding AC-dependence of PKA activation by mushroom body co-stimulation with octopamine. However, the downstream effects of the AC–cAMP–PKA cascade remained clouded. We here suggest that, similar to the situation in snails (Hümara et al. 2004), one of these PKA effectors is synapsin, such that synapsin phosphorylation allows a transient recruitment of synaptic vesicles from the reserve pool to the readily releasable pool. A subsequent presentation of the learned odor could then draw upon these newly recruited vesicles. This scenario also captures the lack of additivity of the syn97 and rut2080 mutations in adult odor–shock associative function, and the selective defect of the syn97-mutation in short-rather than longer-term memory (Knapet el al. 2010).

Given that the memory trace established in our paradigm likely is localized to few cells relative to the brain as a whole (see following section), given that these are transient, short-term memory traces (Neuser et al. 2005), and given the possibility of dephosphorylation, it is not unexpected that Nuval et al. (2011) have not uncovered either predicted PKA site of synapsin as being phosphorylated in a biochemical approach, using whole-brain homogenates from untrained adult Drosophila (for similar results in Drosophila embryos, see also Zhai et al. 2008). Given the likely spatial and temporal restriction of these events in vivo, immunohistological approaches are warranted to see whether, where, and under which experimental conditions synapsin phosphorylated at either of its PKA sites indeed can be detected.

Interestingly, the evolutionarily conserved N-terminal PKA-1 site undergoes ADAR-dependent mRNA editing (Diegelmann et al. 2006), which despite the genomically coded RRFS motif yields a protein carrying RGFS. This editing event, as judged from whole-brain homogenates, occurs for most but not all synapsin and, as suggested by in vitro assays of an undecapeptide with bovine PKA, may reduce phosphorylation rates by PKA. Given that the successfully rescuing UAS–syn construct (Figs. 2, 3) codes for the edited RGFS sequence, it should be interesting to see whether this rescue is conferred by residual phosphorylation at PKA-1, and/or by phosphorylation of the evolutionarily non-conserved PKA-2 site. Last, but not least, one may ask whether an otherwise wild-type synapsin protein featuring a nonedited RRFS motif is also rescuing associative function.

In any event, our finding that the PKA consensus sites of synapsin are required to restore learning in the syn97-mutant (Fig. 2E vs. Fig. 6E,L) is the first functional argument to date, in any experimental system, to suggest synapsin as an effector of the AC–cAMP–PKA cascade in associative function.

Cellular site: A memory trace in the projection neurons?
In contrast to our current results in larvae, Thum et al. (2007) argue that not only the mushroom bodies but also projection neurons accommodate appetitive short-term memory traces in adult Drosophila (for the situation in bees, see also Menzel 2001). How can this be reconciled?

- **Projection neurons may house such a memory trace in adults, but not in larvae.** However, despite the reduced cell number in larvae, the general layout of the olfactory system appears strikingly similar to adults (Gerber et al. 2009).
- **A projection neuron memory trace may be rutabaga-dependent, but synapsin-independent.** As rutabaga and synapsin are present within most if not all neurons, with rutabaga arguably acting...
Figure 6. No rescue by a synapsin protein with mutated PKA sites. The upper panel shows the organization of transgenically expressed SynapsinPKA-AlaAla with both PKA sites mutated. (A–D, G–K) Anti-synapsin (white) and anti-F-actin (orange) immunoreactivity of brains of the indicated genotypes. (E) Expression of synapsin with mutated PKA sites does not rescue associative function in syn97-mutant larvae. (F) No haploinsufficiency caused by the the UAS–synPKA-AlaAla insertion. (G–M) Using an independent EFFECTOR fly strain, with the UAS–synPKA-AlaAla construct inserted at a different site, yields the same results. Scale bars: 50 μm. All other details as in the legend of Figure 1. (N) Working hypothesis of the molecular mode of synapsin action in associative learning. Our results suggest a memory trace for the association between odor and reward to be localized within the Kenyon cells (KC). The type I adenylyl cyclase (AC) acts as a molecular coincidence detector: the odor leads to presynaptic calcium influx, and hence to an activation of calmodulin, whereas the reward leads to an activation of likely octopaminergic neurons and the corresponding G-protein coupled receptors (Hauser et al. 2006). Only if both these signals are present, the AC–cAMP–PKA cascade is triggered, and the respective effector proteins, including synapsin, are phosphorylated. This allows a recruitment of synaptic vesicles from the reserve pool to the readily releasable pool. Upon a subsequent presentation of the learned odor, more transmitter can be released (Hilfiker et al. 1999). This strengthened output is proposed to mediate conditioned behavior towards the odor at test.
upstream of synapsin (Fig. 6N), this would need to assume that the AC–cAMP–PKA cascade is specifically disconnected from synapsin in the projection neurons.

- The rutabaga rescue in projection neurons may be nonassociative. Appetitive training may nonassociatively increase the gain of all projection neuron-to-mushroom body synapses, and this may be rutabaga-dependent. As rutabaga expression in the projection neurons rescues associative performance, however, one would need to additionally assume that residual rutabaga function in the mushroom bodies of the rut2080 mutants (the rut2080 allele is not a null-allele) (Pan et al. 2009) is only able to support an associative memory trace in the mushroom bodies if the mushroom bodies are driven sufficiently strong, by virtue of the nonassociative facilitation of their input. This would integrate two further observations that argue against a functionally independent, appetitive associative short-term memory trace in the projection neurons: (1) expression of a constitutively active Gw, in only the mushroom body impairs adult odor–sugar learning (Thum 2006; loc. cit. Fig. 13). (2) Blocking projection neuron output during training prevents appetitive associative memory formation (H Tanimoto, unpubl.).

- We may have overlooked a projection neuron rescue. (1) As argued above (Fig. 4F,M), a haploinsufficiency caused by the GH146–Gal4 and NP225–Gal4 insertions can be ruled out as reason for such inadvertence. (2) Both employed odors may be processed only outside the covered projection neurons. Thus, blocking synaptic output from these neurons should leave olfactory behavior unaffected—we find, however, that odor preferences in such an experiment are massively reduced (for NP225–Gal4; Supplemental Fig. S2). (3) Within the subset of covered projection neurons, the activity patterns evolved by both odors may actually be the same. Discrimination between them may rely on between-odor differences outside of covered projection neuron subset. However, even in a one-odor paradigm, which does not require discrimination between two odors, we find no projection neuron rescue either (Fig. 4N–P).

- Adult rutabaga expression by GH146–Gal4 and NP225–Gal4 may include neurons that are not covered in the larva. A careful assessment of anti-rutabaga immunohistochemistry is a prerequisite to see whether this is the case.

- Adults, but not larvae, need to be starved before appetitive learning, such that a discrepancy between larvae and adults may be affected by motivational differences.

To us, none of these scenarios seems fully compelling; it therefore appears that for the time being it must remain unresolved whether indeed there is a discrepancy between larvae and adults regarding a projection neuron memory trace, and if so, why this would be the case. In any event, from the present data on the larva, a synapsin-dependent memory trace in the projection neurons does not need to be reckoned with.

**Cellular site: A role for mushroom body subsystems?**

Are the mushroom bodies necessary for olfactory associative function in larvae, as is arguably the case in adults (reviewed in Gerber et al. 2009)? Heisenberg et al. (1985) found that the mmb1 mutation, which causes miniaturized mushroom bodies, is strongly impaired in an odor–electric shock associative paradigm. Twenty-five years later, Pauls et al. (2010) reported that blocking synaptic output of mushroom body neurons by means of shibire expression throughout training and testing reduces odor–sugar associative function. Interestingly, this effect differed between driver strains used. Using GFP expression as a stand-in for shibire expression and assuming that all mushroom body neurons are sensitive to the effects of shibire, Pauls et al. (2010) argued that intact output from specifically embryonic-born mushroom body neurons is necessary for associative function. In turn, embryonic-born mushroom body neurons are apparently sufficient for associative function, as already stage one larvae, not yet equipped with larval-born mushroom body neurons, can perform in the task, and because ablating larval-born mushroom body neurons by means of hydroxy urea treatment was without effect. Thus, embryonic-born mushroom body neurons appear sufficient, and intact synaptic output from them required, for proper odor–reward associative function in the larva.

Our present analysis shows that restoring synapsin in the mushroom bodies is sufficient to fully restore associative function. Strikingly, expression of synapsin in only a handful of mushroom body neurons is sufficient in this regard (Fig. 3K; using DS2H–Gal4). Despite the low number of covered cells, the majority of the 36 mushroom body-gomerali appear innervated (Masuda-Nakagawa et al. 2005, 2009). Indeed, Masuda-Nakagawa et al. (2005) showed that each mushroom body neuron on average receives input in a random subset of six from the total ~36 gomerali. Thus, if more than six randomly chosen mushroom body neurons are included by a Gal4 strain, fairly broad aspects of the olfactory input space should be covered (see also Murthy et al. 2008). We note, however, that the DS2H–Gal4 element includes a *dunce* enhancer sequence (Qiu and Davis 1993). The *dunce* gene codes for a CAMP-specific phosphodiesterase required for associative function in adult and larval *Drosophila* (Aceves-Pina and Quinn 1979; Tully and Quinn 1985) and is expressed in the mushroom bodies of both stages (Nighorn et al. 1991). Thus, it may be that these neurons are of peculiar role for establishing a memory trace.

Our present analysis, with an important caveat, also suggests a requirement of the mushroom bodies. Restoring synapsin throughout the brain, but excluding the mushroom bodies, does not restore associative function (Fig. 5). The caveat, however, is that global synapsin expression (by elav–Gal4) with an intended local repression in the mushroom bodies (by mb247–Gal80) apparently reduces synapsin expression also outside the expression pattern expected from the mb247-element (an effect that can unwittingly be overlooked if using GFP expression as stand-in for the experimental agent) (Fig. 5G,H). Unfortunately, an independent assault toward necessity, namely, to locally reduce synapsin expression by RNAi, does not appear feasible, as we could not document an actual local reduction of synapsin expression in larval mushroom bodies in whole mount brains, likely because mushroom body neurons expressing the transgene are too closely intermingled with mushroom body neurons that do not (not shown).

**Outlook**

We have identified the mushroom bodies (Fig. 3), but not the projection neurons (Fig. 4), as a cellular site of action of synapsin in odor–sugar associative function of larval *Drosophila*. We provide experimental evidence to suggest that the molecular mode of action of synapsin is as a substrate of the AC–cAMP–PKA pathway (Fig. 6). This analysis brings us closer toward an unbroken chain of explanation from the molecular to the cellular level and further to a learned change in behavior. Given the homology of many of the molecular determinants for synaptic and behavioral plasticity (Pittenger and Kandel 2003; Davis 2005) this may become relevant for biomedical research. Last but not least, on the cellular level, an understanding of which specific sites along a sensory–motor circuit are altered to accommodate behavioral changes may be inspiring for the design of “intelligent” technical equipment.
Materials and Methods

Third-instar feeding-stage larvae aged 5 d after egg laying were used throughout. Animals were kept in mass culture, maintained at 25°C (unless mentioned otherwise), 60%–70% relative humidity and a 14/10-h light/dark cycle. Experimenters were blinded with respect to genotype and treatment condition in all cases; these were decoded only after the experiments.

Fly strains
We used the wild-type CS strain (Michels et al. 2005) as reference throughout. The syn97/CS mutant strain, carrying a 1.4-kb deletion in the synapsin gene and lacking all synapsin, had been outcrossed to wild-type CS for 13 generations (Godenschwege et al. 2004; Michels et al. 2005) and will be referred to as syn97 for simplicity.

In all cases when transgenic strains were involved, these strains were all on the w1118-mutant background and carry a miniature rescue construct on their respective transgene to keep track of those transgenes. The w1118 mutation is without effect in our associative learning paradigm (Figs. 1K, 4F,M; see also Yarali et al. 2009).

Driver and effector strains
We recombined various transgenic Gal4 driver strains into the syn97-mutant background by classical genetics (roman numerals refer to the chromosome carrying the construct):

- elav–Gal4; syn97 [X] (C155 in Lin and Goodman 1994) for brain-wide transgene expression;
- mb247–Gal4; syn97 [III] (Zars et al. 2000) for transgene expression in many mushroom body neurons;
- D52I–Gal4; syn97 [X] (Qi and Davis 1993; Tettamanti et al. 1997) (kindly provided by R. Davis, Baylor College, Houston), for transgene expression in a small subset of mushroom body neurons;
- GH146–Gal4; syn97 [II] (Heimbeck et al. 2001) for transgene expression in projection neurons;
- NP223–Gal4; syn97 [II] (Tanaka et al. 2004) also for transgene expression in projection neurons.

As effector strains we used the transgenic UAS–syn; syn97 [III] strain (generated on the basis of Lohr et al. 2002), a UAS–RNAi–syn [III] strain (see below), or UAS–shi61 [III] to block neurotransmitter release (Kitamoto 2001).

Rescue
Three kinds of crosses were performed, of flies all in the w1118 mutant background:

- RESCUE: we crossed a homozygous driver strain, e.g., elav–Gal4; syn97 to a homozygous UAS–syn, syn97 effector strain, yielding double heterozygous larvae, in the synapsin–mutant background: elav–Gal4/+; UAS–syn, syn97/syn97;
- DRIVER control: we correspondingly crossed e.g., elav–Gal4; syn97 to syn97 yielding single-heterozygous elav–Gal4/+; syn97/syn97;
- EFFECTOR control: we crossed UAS–syn, syn97 to syn97 yielding single-heterozygous; UAS–syn, syn97/syn97.

When other expression patterns were desired, the respective other Gal4 strains were used.

Excluding the mushroom bodies from the rescue-expression pattern
To restore synapsin expression throughout the brain, but not in the mushroom body, a mb247–Gal80; UAS–syn, syn97 effector strain was generated (generous gift from S. Knapek) by classical genetics from mb247–Gal80 [II] (Krashes et al. 2007) and UAS–syn, syn97 (see above). Because Gal80 is an inhibitor of Gal4, Gal80 can suppress Gal4 in the mushroom body and thus prevent synapsin expression in the mushroom bodies. The following crosses were performed, of flies all in the w1118 mutant background:

- No-mushroom body RESCUE: flies of the mb247–Gal80; UAS–syn, syn97 effector strain were crossed to elav–Gal4; syn97 as driver strain. This yielded triple-heterozygous elav–Gal4/+; mb247–Gal80/+; UAS–syn, syn97/syn97;
- DRIVER control: we crossed elav–Gal4; syn97 to syn97 yielding elav–Gal4/+; syn97/syn97;
- EFFECTOR control: we crossed mb247–Gal80; UAS–syn, syn97 to syn97 yielding; mb247–Gal80/+; UAS–syn, syn97/syn97.

Induced rescue
For induced expression of synapsin, we generated a fly strain carrying tub–Gal80P [II] (McGuire et al. 2003) and UAS–syn in the mutant background (tub–Gal80P; UAS–syn, syn97). The following crosses were performed, of flies all in the w1118 mutant background:

- Induced brain-wide RESCUE: tub–Gal80P; UAS–syn, syn97 flies were crossed to elav–Gal4; syn97 to yield elav–Gal4/+; tub–Gal80P/+; UAS–syn, syn97/syn97;
- DRIVER control: elav–Gal4; syn97 was crossed to syn97 yielding elav–Gal4/+; syn97/syn97;
- EFFECTOR control: we crossed tub–Gal80P; UAS–syn, syn97 to syn97 yielding; tub–Gal80P/+; UAS–syn, syn97/syn97.

These crosses were cultured at 18°C. To induce synapsin expression, a 30°C HS was applied for 24 h on day 6 AEL. Then, vials were kept at room temperature for 2 h before experiments were performed. Thus, synapsin expression is expected only in the induced brain-wide RESCUE strain and only when an HS was applied. This is because Gal80P suppresses Gal4-mediated transgene expression at 18°C but not at 30°C.

RNAi
To yield an RNAi-mediated knock-down of synapsin, a UAS–RNAi–syn [III] strain was generated. A 497 nt coding fragment of the syn–CDNA was amplified by PCR with primers containing unique restriction sites: the primer pair 5′-GAGCTCTTGAAC CGATGCAGAGATGCTCTG-3′ and 5′-GAGCCATTTCTGCGGCGTGC TGATC-3′ was used for the cDNA fragment and 5′-GAATTCGCCCGCTGCCGCTGCTC-3′ and 5′-GAGC GAATTCCGCGCTGCGCGTCTC-3′ were used for the antisense cDNA fragment, respectively. The PCR-amplified fragments were digested with XbaI/EcoRI and EcoRI/KpnI, respectively, and subcloned into XbaI/KpnI pBluescript KSII (Stratagene). The resulting inverted repeat sequence was excised as a 1-kb NotI/KpnI fragment, ligated into NotI/KpnI-cut pUAST (Brand and Perrimon 1993) and transformed into UAS–RNAi-dependent Sure2 supercompetent cells (Stratagene). Germ-line transformation was performed into a w1118 strain (Bestgene). For experiments, the following crosses, all in the w1118 mutant background, were performed:

- KNOCK-DOWN: UAS–RNAi–syn was crossed to UAS–dcr-2; elav–Gal4 (generated by classical genetics from the UAS–dcr-2 [X] strain [Dietz et al. 2007] and the elav–Gal4 [III] strain, both from Bloomington stock center); this yielded triple-heterozygous animals of the genotype UAS–dcr-2/+; elav– Gal4/UAS–RNAi-syn.
- DRIVER control: we crossed UAS–dcr-2; elav–Gal4 to no-transgene carrying flies yielding UAS–dcr-2/+; elav–Gal4/+;
- EFFECTOR control: we correspondingly generated; UAS– RNAi-syn/+.  

Expression of mutated transgenes

In order to generate loss-of-function mutations in both putative PKA phosphorylation sites of synapsin, site-directed mutagenesis was performed (see sketch in Fig. 6). The syn-cDNAs containing SerPKA-1 → Ala and SerPKA-2 → Ala were amplified by PCR using the following primers: for amplifying the nonphosphorylatable PKA-1, the primer pair Ser → Ala PKA 1 forward, 5'-GAGCT CTCCCGAGGCGAATCCTCT-3' and Ser → Ala PKA 1 reverse 5'-GGATCC GACTCGTACACTACTG-3' and Ser → Ala PKA 2 forward 5'-GGATCC AGTGGTGGTGGTGGGCTGTGATGCGAAGAACTAGT-3' and Ser → Ala PKA 2 reverse 5'-GGGAACAAAGTGGTGGTGGGCTGTGATGCGAAGAACTAGT-3'. The PCR-amplified fragments were digested with SpeI and PstI and subcloned successively into SpeI-PstI-digested pBluescript KSII vector (Stratagene) containing the syn-cDNA over EcoRl, and sequenced. The resulting mutated syn-cDNA sequence was excised as a 3.4-kb EcoRl fragment, ligated into the EcoRl-cut pUAST vector (Brand and Perrimon 1993) and transformed into recombinant-deficient TOPI10 (in vitro) chemically competent Escherichia coli cells (Invitrogen GmbH). Germ-line transformation then was performed into the w1118 ∆(Scribes) strain (Bestgene), yielding two effector strains, namely, UAS–synPKA-AlaAla, syn97, yielding the synPKA-AlaAla strain, and SerPKA-AlaAla, syn97, yielding the synPKA-AlaAla strain. The latter strain is an independent insertion strain of the same UAS–synPKA-AlaAla construct. The following genotypes could thus be generated:

- RESCUEPKA-AlaAla, UAS–synPKA-AlaAla, syn97 flies were crossed to elav–Gal4; syn97, resulting in double heterozygous elav–Gal4/++; UAS–synPKA-AlaAla, syn97/+ synPKA-AlaAla larvae;
- DRIVER control: we correspondingly crossed elav–Gal4; syn97 to syn97 yielding single heterozygous elav–Gal4/++; syn97/syn97;
- EFFECTOR control: we crossed UAS–synPKA-AlaAla, syn97 to syn97 yielding; UAS–synPKA-AlaAla, syn97/syn97.

Western blotting

For each lane in the Western blots, 10 larval brains were homogenized in 10 μl 2 × SDS gel loading buffer. The sample was heated to 70°C for 5 min and centrifuged for 2 min before electrophoresis. Proteins were separated by 12.5% SDS-PAGE in a Multigel chamber (100 mA, 3 h; PEQLAB) and transferred to nitrocellulose membranes (Kyhse-Andersen 1984). Immunoreactions were visualized with two monoclonal mouse antibodies: SYNORF1 for synapsin detection (Klagges et al. 1996) (dilution 1:100), and ab49 (Zinsmaier et al. 1990, 1994) (dilution 1:400) for detection of the Cysteine String Protein (CSP) (Arnold et al. 2004) as loading control. Visualization was achieved with the ECL Western blot detection system (Amersham, GE Healthcare).

Immunohistochemistry

Larval brains were dissected in phosphate-buffered saline containing 0.3% Triton X-100 (PBST) and fixed in 4% paraformaldehyde dissolved in PBST for 1 h. After three washes (each 10 min) in PBST, the brains were treated in blocking solution containing 3% normal goat serum (Dianova) in PBST for 1.5 h. Tissue was then incubated overnight with the primary monoclonal anti-synapsin mouse antibody (SYNORF1, diluted 1:10 in blocking solution) (Klagges et al. 1996). Six washing steps in PBST (each 10 min) were followed by incubation with a secondary rabbit anti-mouse antibody conjugated with Alexa 488 (diluted 1:200) (Molecular Probes; Lot 41A1-4), which visualizes filamentosus actin. After final washing steps with PBST, samples were mounted in Vectashield (Linaris).

In cases when we sought for an independent approximation of transgene expression supported by the various driver strains, we crossed the respective driver strains to UAS–mCD8::GFP flies (labeled as UAS–GFP for simplicity throughout) (Lee and Luo 1999) and probed for GFP expression. To this end, larval brains were incubated with a primary polyclonal rabbit anti-GFP serum (A11126; Invitrogen). After washing with PBST, samples were incubated with a secondary goat antirabbit serum (Alexa Fluor 488, antirabbit lg, diluted 1:100) (MobiTech).

Three-dimensional reconstructions of larval brain stainings were accomplished with the ImageJ 3D Viewer and Segmentation Editor (Schmid et al. 2010).

Scanning electron microscopy (SEM)

For SEM, larvae were collected in water and cooled to immobility for 30 min. The last third of the animal was cut off and larvae were fixed overnight in 6.25% glutaraldehyde with 0.05 mol 1 l-1 Sørensen phosphate buffer (pH 7.4). Fixed specimens were washed five times in buffer for 5 min each and dehydrated through a graded series of acetone. After critical-point drying in CO2 (BALTEC CPD 030), larvae were mounted on a table and sputtered with Au/Pd (BALTEC SCD 005). Specimens were viewed using a scanning electron microscope (Zeiss DSM 962).

Associate learning experiments

Learning experiments follow standard methods (Scherer et al. 2003; Neuser et al. 2005; for a detailed protocol see Gerber et al. 2010) (sketch in Fig. 1C), employing a two-odor, reciprocal conditioning paradigm, unless mentioned otherwise. In brief, olfactory choice performance of larvae was compared after either of two reciprocal training regimes: During one of these regimes, larvae received n-amylacetate (CAS: 628-63-7; AM; Merck) as an associative reward (+) and 1-octanol (CAS: 111-87-5; OCT; Sigma-Aldrich) without reward (AM+/OCT); the second regime involved reciprocal training (AM+/OCT). Then, animals were tested for their preference between AM and OCT. Associative learning is indicated by a relatively higher preference for AM after AM+/OCT training compared to the reciprocal AM/OCT + training (behavioral paradigms not using such a reciprocal design [Honjo and Furukubo-Tokunaga 2005; Honjo and Furukubo-Tokunaga 2009] can be confounded by nonassociative effects [Gerber and Stocker 2007] and are therefore not discussed throughout this paper). These differences in preference were quantified by the associative performance index (PI; see below).

Petri dishes (Sarstedt) with 85-mm inner diameter were filled with 1% agarose (electrophoresis grade; Roth), allowed to solidify, covered with their lids, and, at room temperature, left untreated until the following day. As reward we used 2 mol fructose (FRU, purity: 99%; Roth) added to 1 l of agarose.

Experiments were performed in red light under a fume hood at 21°C–24°C. Before experiments, we replaced the regular lids of the Petri dishes with lids perforated in the center by 15 1-mm holes to improve aeration. A spoonful of food medium containing larvae was taken from the food bottle and transferred to a glass vial. Thirty animals were collected, washed in tap water, and transferred to the assay plates. Immediately before a trial, two containers loaded both with the same odor had been placed onto the assay plate on opposite sides of the plate. Within each reciprocal training condition, for half of the cases we started with a different odor and to the respective other substrate for 5 min. This cycle was repeated three times.

For testing, the larvae were placed in the middle of a fresh assay plate which did not contain the reward. One container of AM was placed on one side and one container of OCT on the other side. After 3 min, the number of animals on the “AM” or “OCT” side was counted. Then, the next group of animals was trained reciprocally. For both reciprocally trained groups, we then calculated an odor preference ranging from –1 to 1 as the number of
animals observed on the AM side minus the number of animals observed on the OCT side, divided by the total number of animals:

\[
\text{PREF} = \frac{(#\text{AM} - #\text{OCT})}{#\text{TOTAL}}
\]

(1)

For all learning experiments, these PREF values are documented in the Supplemental Material (Supplemental Fig. S3).

To determine whether these preferences are different depending on training regimen, we calculated an associative performance index ranging from -1 to 1 as:

\[
\text{PI} = \frac{\text{PREF}_{\text{AM}} - \text{PREF}_{\text{OCT}}}{\text{PREF}_{\text{AM/OCT}}}/2
\]

(2)

After data for one such index for one genotype was collected, data for the next genotype of the respective experiment were gathered; that is, all genotypes to be compared statistically were run side by side (in temporal “parallelity”).

Statistical analyses

We displayed the PI scores as box plots (middle line: median; box boundaries and whiskers: 25%/75% and 10%/90% quantiles, respectively). For statistical comparisons, we used nonparametric statistical analyses throughout (multiple-genotype comparisons: Kruskal-Wallis [KW] tests; two-genotype comparisons: Mann-Whitney U-tests [MW]). To retain an experiment-wise error of 5% in cases of multiple tests, the significance level was adjusted by a Bonferroni correction, i.e., by dividing 0.05 by the number of the respective tests. All calculations were performed with Statistica 7.1 (StatSoft Inc.) on a PC.

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