Research

EGFR signaling in the brain is necessary for olfactory learning in *Drosophila* larvae

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Signaling via the epidermal growth factor receptor (EGFR) pathway has emerged as one of the key mechanisms in the development of the central nervous system in *Drosophila melanogaster*. By contrast, little is known about the functions of EGFR signaling in the differentiated larval brain. Here, promoter-reporter lines of EGFR and its most prominent activating ligands, Spitz, Keren, and Vein, were used to identify the brain structures relevant for the EGFR pathway. Unexpectedly, promoter activity of all these pathway components was found in the mushroom bodies, which are known to be a higher brain center required for olfactory learning. We investigated the role of the EGFR pathway in this process by using different mutant larvae with reduced pan-neuronal EGFR signaling and those with reduced EGFR signaling in mushroom bodies only. Expression of a dominant-negative form of EGFR as well as silencing of the ligands via RNA interference was applied and resulted in significantly impaired olfactory learning performances. General defects in the ability to taste or smell as well as impaired EGFR signaling during embryonic development could be excluded as major reasons for this learning phenotype. In addition, targeted expression of a constitutively active form of the ligand Spitz also led to a significantly reduced learning ability. Thus, very low levels as well as very high levels of EGFR signaling are deleterious for olfactory learning and memory formation. We hypothesize that EGFR signaling in a certain range maintains a homeostatic situation in the mushroom bodies that is necessary for proper learning and memory.

[Supplemental material is available for this article.]

Epidermal growth factor receptor (EGFR) signaling is highly conserved throughout the animal kingdom (Stein and Staros 2006; Sibilia et al. 2007; Fraguas et al. 2011). As one of the major signaling pathways involved in tumor growth and metastasis, it has been extensively studied in mammalian cells and numerous animal models (Arteaga 2006; Hambardzumyan et al. 2011; Mimeault and Batra 2011; Read 2011). Excessive EGFR signaling has been causally linked to the development of various cancers (Moasser 2007; Harris and McCormick 2010; Hatanpaa et al. 2010). By contrast, reduced signaling in the brain has recently been associated with cognitive impairment in Alzheimer's and Parkinson's disease (Chen-Plotkin et al. 2011; Hochstrasser et al. 2012).

Drosophila is well suited to study the molecular basis of human neurological diseases (Bonner and Boulianne 2011; Whitworth 2011) because complex genetic manipulations can be performed to analyze the functions of neurons and neuronally expressed genes in vivo (Venken et al. 2011). Moreover, compared to the situation in mammals, the molecular repertoire of phylogenetically conserved signaling pathways, such as the EGFR pathway, is much simpler in *Drosophila* (Shilo 2003). At the same time, the nervous systems of mammals and *Drosophila* share several remarkable similarities, which is particularly true for the olfactory system (Davis 2004; Sato and Touhara 2009). Thus, olfactory classical conditioning assays are most commonly used in *Drosophila* larvae and adult flies to quantify learning and memory abilities (Selcho et al. 2009; Akalal et al. 2010). According to classical conditioning, different odors are presented in these assays as conditional stimuli that must be associated with aversive

(punishment) or appetitive (reward) stimuli, which are, in turn, defined as unconditional stimuli (Pavlov 1927).

The mushroom bodies (MBs), a specialized region of the insect brain, are essential to associate conditional and unconditional stimuli and therefore are considered the memory centers in adult flies and larvae (Sato and Touhara 2009; Busto et al. 2010; Pauls et al. 2010). Within MB neurons, the adenylyl cyclase (AC) rutabaga is assumed to function as a coincidence detector capable of associating information from both stimuli. This enzyme can be activated synergistically by odor-induced Ca^{2+} and by taste-induced G protein-coupled receptor signaling (Gervasi et al. 2010). Subsequent cAMP/PKA pathway activation plays a crucial role in synaptic plasticity and memory storage (Blum et al. 2009; Tomchik and Davis 2009).

However, the possible role of the EGFR pathway in memory function has not yet been investigated. To test whether EGFR-mediated signaling is physiologically important in the brain, we first generated promoter-*gfp* (green fluorescent protein) reporter lines for several pathway components. Unexpectedly, microscopic analyses revealed predominant expression of EGFR and its ligands Spitz, Keren, and Vein in the larval MBs. Subsequently, we used an inducible dominant-negative strategy and RNAi-mediated silencing to specifically impair EGFR pathway activation in the central nervous system (CNS) during larval stages. By employing these animals in olfactory classical conditioning assays, we demonstrated that EGFR signaling is essential for olfactory learning and memory in *Drosophila* larvae.

Results

EGFR signaling components are expressed in larval MBs

To analyze the expression of EGFR pathway components, we generated promoter-*gal4* lines for EGFR and its most prominent

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ligands *Spitz*, *Keren*, and *Vein*. These strains were crossed with a UAS-mCD8::GFP (membrane-bound GFP) line, and immunohistochemistry was performed on whole-mount L3 larval and adult brains of the F₁ generation. GFP expression was detected using anti-GFP immunohistochemistry. In the adult CNS, no promoter activity of *egfr* was observed. Of the ligand genes, only *spitz* and *keren* showed promoter activity in adult MBs (data not shown). However, in the brain lobes of the larval nervous system, we found distinct promoter activity of the *egfr* gene, which was restricted to the MBs (Fig. 1A). Moreover, the reporter lines for the three ligand genes *spitz*, *keren*, and *vein* (Fig. 1B–D) also showed promoter activity in the larval brain lobes. Here, again the MBs exhibited the strongest GFP signals (Fig. 1B–D). This observation suggests that EGFR-mediated signaling is of high physiological importance in the larval MBs. As these structures are known as centers for olfactory learning and memory, we performed olfactory learning experiments with larvae with impaired neuronal EGFR signaling.

Impaired olfactory learning in larvae constitutively expressing a dominant-negative EGFR

Olfactory learning ability was investigated in larvae characterized by pan-neuronal EGFR signaling manipulation. Using pan-neuronal driver lines ensures that all brain structures relevant for memory formation are targeted. The effect of constitutive and pan-neuronal expression of a dominant-negative isoform of EGFR (*nsyb-gal4* > UAS-EGFR^{DN}) compared to that of matching controls (*nsyb-gal4* > *w¹¹¹⁸*) was analyzed in the olfactory learning en mass assay (Neuser et al. 2005). Control larvae demonstrated a significantly higher learning index compared to those expressing dominant-negative EGFR (Fig. 2A, $P = 0.0022$, $n_{\text{learning indices}} = 6$, $n_{\text{larvae}} > 170$). The median learning index of the experimental

Figure 2. Constitutive and pan-neuronal EGFR^{DN} expression impairs larval learning. (A,B) F₁ third instar control larvae (*nsyb-gal4* > *w¹¹¹⁸*) were compared with those with constitutively impaired pan-neuronal EGFR signaling (*nsyb-gal4* > UAS-EGFR^{DN}). Larvae were tested in an en mass assay ($n_{\text{learning indices}} = 6$ each; $n_{\text{larvae}} > 170$ each) (A) and in an individual-animal assay ($n_{\text{learning indices}} = 64$ each) (B). (C) Scheme of the olfactory learning assays. In the en mass assay, groups of about 15 larvae were trained and tested together. After 3 min in an odor-choice situation between AM and OCT, the number of larvae on the different odor sides was counted. In the individual-animal assay, a group of eight larvae was trained together but tested individually to exclude nonindependent behavior. (AM) Amyl acetate, (FRU) fructose, (OCT) 1-octanol, (QUI) quinine, (***) $P < 0.01$, (**) $P < 0.001$.

group (0.2) was reduced to 67% of that of the control group (0.3). To exclude nonindependent confounding interactions between individuals, larvae were also tested individually using an individual-animal version of the learning assay (Scherer et al. 2003). As shown in en mass assays, control larvae had a significantly better learning capability than those expressing dominant-negative EGFR (Fig. 2B, $P < 0.0001$, $n_{\text{learning indices}} = 64$ each). The median learning index of the control larvae, 0.4, clearly indicated olfactory learning, whereas the median learning index of larvae expressing EGFR^{DN} was reduced to 0.

Constitutive and pan-neuronal expression of EGFR^{DN} does not impair taste or smell

Olfactory learning deficits may result from general defects in the ability to taste or smell. To exclude this possibility, naive, untrained larvae were tested for their response to gustatory stimuli and to an olfactory attractant. The gustatory capability of control larvae and of larvae constitutively and pan-neuronally expressing EGFR^{DN} was investigated by offering fructose (FRU, attractive taste) or quinine (QUI, repellent taste) compared to pure agarose. Both control larvae and those constitutively and pan-neuronally expressing EGFR^{DN} demonstrated significant preferences for FRU over pure agarose (Fig. 3A) and for pure agarose over QUI (Fig. 3B). Notably, no differences in preferences were detected between these groups of larvae (Fig. 3A, FRU vs. pure, $P = 0.6450$, $n_{\text{Pref}} \geq 20$; Fig. 3B, QUI vs. pure, $P = 0.1026$, $n_{\text{Pref}} \geq 19$).

To test the olfactory abilities of control larvae and larvae constitutively and pan-neuronally expressing EGFR^{DN}, an odor (amyl acetate [AM] or 1-octanol [OCT]) versus no-odor setup was used. Both groups demonstrated significant attraction to both odors (Fig. 3C,D). Importantly, the two genotypes did not reveal any significant difference regarding preferences for the odors

Figure 1. EGFR pathway components are expressed in larval mushroom bodies. Immunostaining of brains of EGFR-gal4 > UAS-gfp larva (A), Spitz-gal4 > UAS-gfp larva (B), Keren-gal4 > UAS-gfp larva (C), and Vein-gal4 > UAS-gfp larva (D). (A–D) Anti-GFP staining indicates expression of the corresponding EGFR pathway genes. Bar, 50 μm.

Figure 3. Constitutive and pan-neuronal EGFR^{DN} expression does not impair taste or smell. (A,B) Gustatory preference (Pref). Control larvae (*nsyb-gal4 > w¹¹¹⁸*) and those constitutively and pan-neuronally expressing EGFR^{DN} (*nsyb-gal4 > UAS-EGFR^{DN}*) showed the same appetitive response to FRU ($n_{\text{Pref}} \geq 20$ each) (A) and aversive response to QUI ($n_{\text{Pref}} \geq 19$) (B). (C,D) Odor sensing. Control larvae (*nsyb-gal4 > w¹¹¹⁸*) and those constitutively and pan-neuronally expressing EGFR^{DN} (*nsyb-gal4 > UAS-EGFR^{DN}*) did not demonstrate any difference in their preference to the odor of AM ($n_{\text{Pref}} = 50$ each) or OCT ($n_{\text{Pref}} = 50$ each). (AM) Amyl acetate, (FRU) fructose, (ns) not significant ($P > 0.05$), (OCT) 1-octanol, (QUI) quinine.

(Fig. 3C, AM vs. no odor, $P = 0.8821$; Fig. 3D, OCT vs. no odor, $P = 0.5978$; $n_{\text{Pref}} = 50$ each). Thus, these results indicate that larvae constitutively and pan-neuronally expressing EGFR^{DN} are impaired in neither taste nor smell. Accordingly, the decreased learning ability of larvae constitutively and pan-neuronally expressing EGFR^{DN} can be attributed to a genuine learning defect.

Pan-neuronal induction of EGFR^{DN} during larval life impairs olfactory learning

To exclude developmental effects, we used the mifepristone-inducible pan-neuronal elav-GeneSwitch line to reduce EGFR signaling exclusively during larval life (Osterwalder et al. 2001). The elav-GeneSwitch line allows us to manipulate gene expression in a time-controlled and temperature-independent manner, making it advantageous for learning experiments with larvae. Control larvae and those treated with mifepristone during larval life were tested in the individual-animal assay. Notably, induced expression of EGFR^{DN} for just 24 h in late larval stages led to significantly reduced olfactory learning ability (Fig. 4A, $P < 0.0001$, $n_{\text{learning indices}} = 64$ each). The control group clearly showed olfactory learning, with a median learning index of 0.4, whereas that of the treated larvae was reduced to 0. Also, this demonstrates that the insertion of the EGFR^{DN} transgene does not affect learning ability. To investigate whether mifepristone itself has an impact on memory formation, elav-GS $> w^{1118}$ control larvae were fed with normal fly food or fly food supplemented with mifepristone for 24 h. Importantly, drug feeding also did not influence the larvae's capability to learn (Fig. 4B, $P = 0.2737$, $n_{\text{learning indices}} = 64$).

In addition, we analyzed the morphology of larval neuropils after induction of EGFR^{DN} for 24 h. Brains of larvae constitutively

and pan-neuronally expressing EGFR^{DN} did not show an altered neuropil morphology when compared to brains of uninduced control larvae (Supplemental Fig. 1). Thus, we assume that reduced olfactory learning capabilities in larvae with impaired pan-neuronal EGFR signaling do not result from disturbances in brain development.

Single EGFR ligands are necessary for olfactory learning

Because not only promoter activity of the *egfr* gene but also that of the activating ligand genes was detected in MBs of the larval brain, we investigated the influence of these upstream components on olfactory learning. For this purpose, the individual-animal assay was performed with larvae expressing pan-neuronal RNAi constructs targeting *spitz*, *keren*, and *vein* during larval stages. In contrast to the induction of EGFR^{DN}, which was performed for only 24 h, the expression of ligand^{RNAi} began immediately after hatching to achieve an adequate silencing effect until experiments were performed with third instar larvae. Again, elavGS $> w^{1118}$ larvae were used in parallel as a control to exclude that drug feeding alone influences learning ability. Comparing the uninduced controls and the induced ligand^{RNAi} animals in the individual-animal assay revealed a significant difference in olfactory learning. RNAi directed against each of the three ligand transcripts led to impaired memory formation (Fig. 5A, *spitz*^{RNAi}, $P < 0.0001$; *keren*^{RNAi}, $P < 0.0001$; *vein*^{RNAi}, $P < 0.0001$; $n_{\text{learning indices}} = 64$ each). The uninduced control larvae of all three ligand^{RNAi} experiments had a median learning index of 0.4 and thus were clearly capable of olfactory learning. The induced ligand^{RNAi} larvae (*spitz*^{RNAi}, *keren*^{RNAi}, or *vein*^{RNAi}) were apparently affected in their ability to learn, exhibiting a median learning index of 0. However, control larvae (elav-GS $> w^{1118}$) that were feeding on fly food supplemented with or without mifepristone did not show any difference in their ability to learn (Fig. 5B, $P = 0.6455$, $n_{\text{learning indices}} = 64$ each). Moreover, the odor sensing as well as the gustatory test was applied to all ligand^{RNAi} larvae to exclude defects in their ability to smell or taste. Uninduced and induced larvae showed the same response to FRU (*spitz*^{RNAi}, $P = 0.8280$; *keren*^{RNAi}, $P = 0.9351$; *vein*^{RNAi}, $P = 0.1469$; $n_{\text{Pref}} = 20$ each) or QUI (*spitz*^{RNAi}, $P = 0.7656$; *keren*^{RNAi}, $P = 0.8071$; *vein*^{RNAi}, $P = 0.7551$; $n_{\text{Pref}} = 20$ each) compared to pure agarose and to AM (*spitz*^{RNAi}, $P = 0.4939$; *keren*^{RNAi}, $P = 0.2768$; *vein*^{RNAi}, $P = 0.8601$; $n_{\text{Pref}} = 50$ each) or OCT (*spitz*^{RNAi}, $P = 0.3040$; *keren*^{RNAi}, $P = 0.4988$; *vein*^{RNAi}, $P = 0.7746$; $n_{\text{Pref}} = 50$ each) compared to no odor (Supplemental Figs. 2, 3). These experiments clearly

Figure 4. Induction of pan-neuronal EGFR^{DN} for 24 h reduces olfactory learning. (A) After pan-neuronal EGFR^{DN} expression for 24 h, olfactory learning ability was significantly impaired in the individual-animal assay ($n_{\text{learning indices}} = 64$ each). (B) An influence of mifepristone on olfactory learning was excluded by feeding normal fly food (ctrl) or fly food supplemented with mifepristone (mife) to elav-GS $> w^{1118}$ control larvae ($n_{\text{learning indices}} = 64$ each). (ctrl) Control, (GS) GeneSwitch, (mife) mifepristone, (ns) not significant ($P > 0.05$), (***) $P < 0.001$.

Figure 5. Induction of pan-neuronal RNAi-mediated silencing of EGFR ligands after hatching leads to decreased olfactory learning ability. (A) In the individual-animal assay, olfactory learning capability was decreased in larvae after induction of pan-neuronal RNAi-mediated gene silencing immediately after hatching ($n_{\text{learning indices}} = 64$ each). (B) Control larvae (*elav-GS > w¹¹¹⁸*) were fed with normal fly food (ctrl) or fly food supplemented with mifepristone (mife) to exclude an influence of the agent on olfactory learning ($n_{\text{learning indices}} = 64$ each). (ctrl) Control, (GS) GeneSwitch, (mife) mifepristone, (ns) not significant ($P > 0.05$), (***) $P < 0.001$.

demonstrate that silencing of a single EGF receptor ligand during post-embryonic stages is sufficient to reduce the learning ability of *Drosophila* larvae.

EGFR signaling balance is important for olfactory learning

Next, we assessed whether memory formation is modified by enhanced EGFR signaling. To address this question, we used the individual-animal assay to investigate larvae that pan-neuronally expressed secreted Spitz (sSpitz) induced for 24 h. These larvae exhibited a clearly reduced olfactory learning ability compared to uninduced controls (Fig. 6, $P < 0.0001$, $n_{\text{learning indices}} = 64$ each). The median learning index of controls was unchanged compared to that of former experiments (0.4), whereas the sSpitz-overexpressing larvae showed only minor learning, with a median learning index of 0.1. Furthermore, sSpitz-overexpressing larvae were not impaired in either taste or smell when applied to the gustatory test (FRU vs. pure, $P = 0.6776$; QUI vs. pure, $P = 0.8284$; $n_{\text{Pref}} = 20$ each) or odor sensing (AM vs. no odor, $P = 0.9945$; OCT vs. no odor, $P = 0.4855$; $n_{\text{Pref}} = 50$ each), respectively (Supplemental Figs. 4, 5).

Olfactory learning is critically dependent on EGFR signaling in the MBs

The promoter activity of EGFR and its ligands Spitz, Keren, and Vein in the larval MBs was the starting point for the investigation of the role of EGFR signaling in olfactory learning. To analyze the relevance of EGFR signaling exclusively in the MBs, we used the driver line OK107-*gal4*, which drives expression predominantly to the MBs, to manipulate the pathway and performed the individual-animal learning assay. The effect of constitutive expression of the dominant-negative isoform of EGFR in the MBs (OK107-*gal4 > UAS-EGFR^{DN}*) on olfactory learning behavior was investigated in comparison to that of matching controls (OK107-*gal4 > w¹¹¹⁸*).

Control larvae had a significantly better learning capability than those expressing EGFR^{DN} exclusively in MBs (Fig. 7, $P < 0.0001$, $n_{\text{learning indices}} = 64$ each). The median learning index of the control larvae, 0.5, clearly indicated olfactory learning, whereas the median learning index of larvae expressing EGFR^{DN} in the MBs was reduced to 0. Notably, the negative effect on learning was comparable with that observed in larvae with pan-neuronal EGFR^{DN} expression.

Discussion

To understand the physiological significance of EGFR signaling in the CNS, we elucidated the expression patterns of EGFR pathway components within the *Drosophila* larval and adult brain using promoter-*gfp* reporter lines. We found that promoter activity of *egfr* and its activating ligands *spitz*, *keren*, and *vein* was most apparent in the larval MBs. In particular, no promoter activity of *egfr* was detected in the adult CNS. Thus, we assumed that EGFR signaling is of high physiological relevance in the differentiated larval brain and, here, is involved in the major task the MBs are known for, learning and memory.

Constitutive and pan-neuronal expression of a dominant-negative form

of EGFR strongly reduced olfactory learning in larvae, whereas their ability to taste and smell was not impaired, indicating that the observed learning phenotype depends on central rather than sensory defects. Moreover, using the inducible, mifepristone-dependent GeneSwitch system, we demonstrated that this learning phenotype results from impaired EGFR signaling in the functional brain and not from developmental defects caused by impaired signaling during embryogenesis. Interestingly, silencing of each of the EGFR ligand genes *spitz*, *keren*, and *vein* at larval stages as well as enhanced expression of secreted (activated) Spitz led to the same dramatic decrease of olfactory learning ability.

Strikingly, reduction of EGFR signaling exclusively in the MBs revealed the same strong learning defect as was observed in larvae with pan-neuronal manipulations of the pathway. This result is in accordance with the prominent promoter activity of EGFR pathway components in the larval MBs and shows that EGFR signaling within the Kenyon cells of the MBs is of critical importance for proper learning abilities.

The most important result of this study is the finding that proper EGFR signaling is essential for olfactory learning in *Drosophila*, particularly with regard to our sparse knowledge of EGFR signaling in the developed brain. In vertebrates, EGFR signaling plays multiple roles during embryogenesis and nervous system development, but has also been linked to myelination (Brinkmann et al. 2008; Luo et al. 2011), Alzheimer's disease (Fleck et al. 2011; Woo et al. 2011), schizophrenia (Harrison and Law 2006), and multiple sclerosis (Aguirre et al. 2007). As in

Figure 6. Pan-neuronal activation of EGFR signaling via secreted Spitz (sSpitz) significantly impairs olfactory learning ability. In the individual-animal assay, olfactory learning capability was significantly decreased in those larvae expressing sSpitz for 24 h compared to the uninduced control larvae ($n_{\text{learning indices}} = 64$ each). (ctrl) Control, (GS) GeneSwitch, (mife) mifepristone, (***) $P < 0.001$.

Figure 7. Constitutive induction of EGFR^{DN} in the larval mushroom bodies reduces olfactory learning. In the individual-animal assay, olfactory learning capability was impaired in those larvae constitutively expressing EGFR^{DN} in the larval MBs (OK107-*gal4* > EGFR^{DN}) compared to control larvae (OK107-*gal4* > w¹¹¹⁸) ($n_{\text{learning indices}} = 64$ each). (***) $P < 0.001$.

vertebrates, little is known about EGFR signaling in the *Drosophila* nervous system. The EGFR pathway has been identified as important for the regulation of sleep (Foltenyi et al. 2007) as well as for ethanol sensitivity (Corl et al. 2009).

A link between EGFR and signaling systems associated with learning was also recently established. Notably, Hannan et al. (2006) connected EGFR signaling to a novel adenylyl cyclase (AC) pathway in the *Drosophila* brain. In their experiments, this neurofibromin 1 (NF1)/Ras-dependent pathway was stimulated by growth factors binding to EGFR to activate an as yet unidentified AC. Furthermore, the pathway is assumed to constitute an alternative to the rutabaga AC-mediated pathway in the brain, with both leading to cAMP/PKA signaling, which is in turn a major component associated with short-term memory formation (Blum et al. 2009). These findings imply that EGFR signaling acts upstream of cAMP/PKA and thus regulates olfactory learning via an alternative AC. Moreover, attenuated as well as enhanced PKA signaling has been reported to reduce learning ability in flies (Drain et al. 1991), which is consistent with our observation that the correct balance of EGFR signaling in the brain is important for memory formation.

Why all three EGFR ligands are essential for proper olfactory learning is not yet understood. One possible explanation is that signaling via all ligands is necessary to maintain the proper signaling balance essential for olfactory learning. Alternatively, the system may not be organized in such a manner that different ligands can be functionally exchanged.

Based on the expression patterns observed for all ligands and EGFR, different modes of signaling are possible. During *Drosophila* brain development, axonal structures are thought to release Spitz to activate the EGFR pathway in glial cells to promote cell survival (Bergmann et al. 2002). Another EGFR ligand, Vein, is produced by pioneer neurons and is required for the survival of longitudinal glia (Hidalgo et al. 2001). Our observations further suggest an alternative signaling system. Because all components of the EGFR signaling system are present in the MBs, autocrine or paracrine modes of signaling are possible. Autocrine EGFR signaling is relevant to various systems ranging from epithelial mechanotransduction (Kojic et al. 2010) to brain tumor development (Tang et al. 1997). Thus, proper autocrine or paracrine EGFR signaling within the Kenyon cells of the MBs may be required to maintain cellular homeostasis, allowing for olfactory learning.

The present findings suggest that holding a fine-tuned homeostatic balance in the cells of the MBs is critical for proper learning. In addition, signaling systems, such as the EGFR pathways that may not be directly involved in transmitting learning related content, may nevertheless be essential to enable learning.

Materials and Methods

Fly strains and conditions

Flies were cultured at 25°C on conventional corn meal, yeast, and glucose agar medium. All RNAi lines, UAS-*spitz*^{RNAi}, UAS-*keren*^{RNAi}, and UAS-*vein*^{RNAi} (Transformant ID 103817, 104299, and 109437, respectively) were obtained from the Vienna *Drosophila* RNAi Center (Austria). The UAS-EGFR^{DN} line (DGRC no. 108189) was from the *Drosophila* Genetics Resource Center (Japan). The UAS-secreted Spitz::GFP line was a gift from Benny Shilo (Rehovot, Israel). Elav-GeneSwitch containing a mifepristone-inducible *elav-gal4* driver was kindly provided by Haig Keshishian (Yale University, USA), an *n-synaptobrevin-gal4* (*nsyb-gal4*) line was a gift from Julie H. Simpson, (Janelia Farm, USA), and the driver line OK107-*gal4* with predominant expression in the MBs was a gift from Dennis Pauls (University of Würzburg, Germany). The 20XUAS-IVS-mCD8::GFP line has the following genotype (w⁺; P[20XUAS-IVS-mCD8::GFP]attP2) and was selected because it shows no Gal4-independent expression in the brain. It was obtained from the Bloomington Stock Center (ID 32194). All other lines were from the Bloomington *Drosophila* Stock Center (USA).

Generation of promoter-*gal4* lines

Presumptive promoter regions (fragments of ~3000–4000 bp located upstream of the translation start) of *spitz* (Dmel/spi, CG10334), *vein* (Dmel/vn, CG10491), *keren* (Dmel/Krn, CG 32179), and *EGFR* (Dmel/Egfr, CG10079) were amplified from genomic DNA using matching oligonucleotides (Biomers):

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pSpitz_fwd attB1CTCGTCTGGGTTTCATCCTG
pSpitz_rev attB2GTAGCGTATCGGCTTGTTTCG
pVein_fwd attB1GATGATACAATGACGCTGCGC
pVein_rev attB2CCAAAGTTGTTGCTGCTATTGC
pKeren_fwd attB1CGTCAATTCGGCTTGAATTGCG
pKeren_rev attB2GGAATTCTTGGCTTTGGCTG
pEGFR_fwd attB1GTAACATCGAACC GGCTGAG
pEGFR_rev attB2CGTTGTGGTGCCTAGTCAAG.
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The amplicons were cloned into pBPGUw (Addgene) using site-specific recombination and subsequently injected into *Drosophila* embryos (Perrimon strain attP40) by BestGene Inc.

Visualizing Gal4 expression patterns by immunofluorescence

To visualize expression driven by the promoter-*gal4* lines, these lines were crossed with the 20xUAS-IVS-mCD8::GFP line. Brains were dissected in 1× PBS, fixed in 4% paraformaldehyde/PBT (1× PBS, 0.3% Triton X-100) for 30 min, washed with PBT, and, after blocking (5% goat serum in PBT) for 30 min, incubated with the primary antibody (rabbit anti-GFP [1:1000, Invitrogen] or mouse anti-nc82 [1:5, Developmental Studies Hybridoma Bank]) in blocking solution at 4°C overnight. After washing with PBT, brains were incubated with the secondary antibody (DyLight488-conjugated goat anti-rabbit or DyLight549-conjugated goat anti-mouse [both 1:400, Jackson ImmunoResearch Europe Ltd.]) in blocking solution at 4°C overnight in the dark. The samples were washed, and embedded in FocusClear (CelExplorer Labs Co.). Fluorescence microscopy was performed with the Axioplan Imager Z1 and Axiocam mRM camera (Carl Zeiss). For Z-projections, the Zeiss ApoTome was used.

GeneSwitch experiments

For experiments with inducible expression of transgenes and RNAi constructs, the elav-GeneSwitch driver line was crossed with the respective UAS-line. A stock solution of 250 µg/mL mifepristone (Sigma-Aldrich) in 50% ethanol was added during fly food preparation to a final concentration of 10 µg/mL. For induction, F₁ generation second-instar larvae were transferred to food

medium supplemented with mifepristone. Larvae placed on food medium without mifepristone but containing the appropriate amount of 2% ethanol served as controls. The olfactory learning experiments were performed with third instar larvae 24 h (± 1 h) after the transfer. For RNAi experiments, eggs were laid on apple juice agar plates overnight and subsequently transferred to mifepristone containing medium or control medium.

Olfactory learning experiments

Olfactory learning experiments were performed at room temperature (21–23°C) according to the method of Scherer et al. (2003), using the odors 1-octanol ([OCT], undiluted, Fluka) and amyl acetate ([AM], diluted 1:50 in paraffin oil, SAFC Supply Solutions) as well as the gustatory reinforcers fructose ([FRU, +], 2 M, Roth) as reward and quinine hemisulfate ([QUI, –], 0.2%, Sigma-Aldrich) as punishment. Lids of 200- μ L PCR tubes (Sarstedt) were utilized for odor presentation.

For the individual-animal assay, a group of eight larvae were trained together but tested individually, whereas for the en mass assay (Neuser et al. 2005) 12–17 larvae were trained and tested together. During the training, an inter-trial phase (Hendel et al. 2005) was inserted between the odor/taste presentations, in which the larvae remained on an empty agarose plate without odors or reinforcers for 1 min. The training cycle was repeated four times (Fig. 2C). During the test phase, the position of the larvae was recorded every 30 sec for a period of 5 min in the individual-animal assay and after 3 min in the en mass assay as “AM,” “OCT,” or “neutral” (a 5-mm-wide zone along the middle of the assay plate). The following formulas were used to estimate learning ability:

$$\text{PREF AM}_{\text{AM+}/\text{OCT-}} = \frac{(\text{number of observations}_{\text{AM}} - \text{number of observations}_{\text{OCT}})}{\text{number of observations}_{\text{total}}}$$

$$\text{PREF AM}_{\text{AM-}/\text{OCT+}} = \frac{(\text{number of observations}_{\text{AM}} - \text{number of observations}_{\text{OCT}})}{\text{number of observations}_{\text{total}}}$$

Subsequently, the learning index was calculated using PREF AM_{AM+ / OCT–} and PREF AM_{AM– / OCT+}:

$$\text{Learning index} = \frac{(\text{PREF AM}_{\text{AM+}/\text{OCT-}} - \text{PREF AM}_{\text{AM-}/\text{OCT+}})}{2}$$

Gustatory test

To test the ability of flies to taste FRU and QUI, a gustatory choice assay was performed according to the method of Hendel et al. (2005). In this assay, experimentally naive larvae can choose between the putative attractive taste of FRU and pure agarose or the putative repellent taste of QUI and pure agarose. Petri dishes (92 mm, Sarstedt) were filled with 1% pure agarose (UltraPure, Invitrogen) on one side and either 2-M FRU or 0.2% QUI in 1% agarose on the other side. Ten larvae were placed along the border of the tasting agent and pure agarose. After 15 min, larvae were recorded as “FRU,” “QUI,” “pure” (without taste), or “neutral” (a 5-mm-wide zone along the middle of the assay plate or moved onto the lid). The preference for FRU or QUI was calculated by subtracting the number of larvae on the “pure” side from the number of larvae on the “taste” side and dividing the difference by the total number of larvae on the plate.

Odor sensing

To analyze the odor sensing ability of larvae, the to-be-associated odors AM or OCT were situated on one side of a Petri dish filled with 1% agarose and were tested versus an empty container. One experimentally naive larva was placed in the middle of the Petri dish and was allowed to move freely for 5 min. The position

of the larva was noted every 30 sec as “AM,” “OCT,” “empty,” or “neutral.” Preference for the odor side was calculated by subtracting the number of counts on the empty side from the number of counts on the odor side and dividing the difference by the total number of counts.

Statistical analysis

The learning index values are represented by box plots (with medians as middle lines, 25% and 75% quantiles as box boundaries, and minimum and maximum values as whiskers). Each box plot of a learning experiment, with the exception of the en mass assay, displays a summary of the results of two biological replicates each performed in duplicate. Preferences for each replicate were sorted in ascending order to calculate the learning indices. Statistical analysis was performed using GraphPad Prism software version 3.0 (GraphPad Software). For comparisons between two groups, the Mann–Whitney test was used for nonparametric data. All learning indices were rounded to one decimal place.

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