The FMRFamide-related neuropeptide FLP-20 is required in the mechanosensory neurons during memory for massed training in \textit{C. elegans}

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Lasting memories are likely to result from a lasting change in neurotransmission. In the nematode \textit{Caenorhabditis elegans}, spaced training with a tap stimulus induces habituation to the tap that lasts for $>24$ h and is dependent on glutamate transmission, postsynaptic AMPA receptors, and CREB. Here we describe a distinct, presynaptic mechanism for a shorter lasting memory for tap habituation induced by massed training. We report that a FMRFamide-related peptide (FMRF = Phe-Met-Arg-Phe-NH$_2$), FLP-20, is critical for memory lasting 12 h following massed training, but is not required for other forms of memory. Massed training correlated with a flip-20-dependent increase in synaptobrevin tagged with green fluorescent protein in the presynaptic terminals of the PLM mechanosensory neurons that followed the timeline of the memory trace. We also demonstrated that flip-20 is required specifically in the mechanosensory neurons for memory 12 h after massed training. These findings show that within the same species and form of learning, memory is induced by distinct mechanisms to create a lasting alteration in neurotransmission that is dependent upon the temporal pattern of training: memory of spaced training results from postsynaptic changes in the interneurons of the neural circuit, whereas memory of massed training results from presynaptic changes in the mechanosensory neurons of the neural circuit.

In this study we found that massed training of tap habituation resulted in a memory that lasted at least 12 h, but not 24 h, after training. We then investigated whether the FMRFamide (Phe-Met-Arg-Phe-NH$_2$)-related peptides (FaRPs) expressed in the six mechanosensory neurons of the tap withdrawal circuit (ALM left and right, AVM, PLM left and right, and PVM) (Chalfie et al. 1985; Wicks and Rankin 1995; Kim and Li 2004) were involved in plasticity of the tap response. FaRPs are found throughout the animal kingdom, and have been shown to act in pain modulation, feeding, and reproduction (Bechtold and Luckman 2006; Kriegsfeld 2006; Li and Kim 2008). The \textit{C. elegans} genome encodes over 70 distinct FaRPs and at least half of the neurons express one or more FaRPs, suggesting that they function in many behaviors (Li and Kim 2008). We found that a FaRP-encoding gene, flip-20, was critical for memory of tap habituation 12 h after massed training, and that this memory was dependent upon a presynaptic mechanism that is distinct from mechanisms known to underlie long-term memory for spaced training.

**Results**

Massed training produces 12-h memory retention that requires a FLP neuropeptide

There is no evidence of memory for habituation in \textit{C. elegans} 24 h after massed training (80 taps presented 60 sec apart) (Rose et al. 2002). Here we tested whether wild-type \textit{C. elegans} would show memory 12 h after massed habituation training. Worms that received massed training (80 taps at a 60 sec interstimulus interval [ISI]) responded with significantly smaller responses to the taps administered during the memory test compared to those of the
rescues the memory defect 12 h after massed training in two independent experiments. Memory was intact in wild-type worms 12 or 24 h later. Interestingly, when they had formed memory of this training.

Figure 1. A mutation in flp-20 that was responsible for tap habituation 12 h after massed training (short-term habituation at a 60 sec ISI was also normal for flp-20 worms; data not shown).

The deficit in 12-h memory following massed training in flp-20 mutants could be rescued by germline transformation of flp-20 mutants with a flp-20 genomic fragment. Two independent rescued lines (two different extra chromosomal arrays), flp-20; ynEx167 [flp-20] and flp-20; ynEx168 [flp-20], showed evidence of memory after massed training (Fig. 1E; F(1,13) = 12.85, P = 0.001 and F(1,4) = 13.05, P = 0.0008, respectively).

Memory of massed training and spaced training are mediated by distinct mechanisms

In C. elegans, memory for tap habituation 24 h after spaced training is dependent on protein synthesis (Beck and Rankin 1995), glutamate signaling (Rose et al. 2002, 2003), and the transcription factor CREB (Timbers and Rankin 2011). To determine whether the flp-20-dependent 12-h memory induced by massed training shared any of these mechanisms, we tested whether disrupting any of the above processes and/or molecules would block memory for tap habituation 12 h after massed training.

To determine whether the 12-h memory retention of massed training is dependent on protein synthesis, we altered protein synthesis by applying heat shock immediately after training (Beck and Rankin 1995; Rose et al. 2002). The cellular response to heat shock (body temperature elevated at least ~2°C–5°C above the optimal level) was first observed in Drosophila and has since been described in every system examined, from bacteria to humans (Schlesinger et al. 1982; Lindquist 1986; Nowak 1993).

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for massed training. Twelve hours post-training, worms with mutations in eat-4, glr-1, or chl-1 showed evidence of memory (Fig. 2B; eat-4 and glr-1, F1,30 = 8.26, P = 0.007 and F12,33 = 5.92, P = 0.02, respectively: Fig. 2C, chl-1, F1,284 = 95.73, P < 0.0001), suggesting that the 12-h memory retention of massed training is not glutamate- or CREB-dependent.

An increase of synaptic vesicles in the presynaptic terminals of PLM follows massed training and is dependent on flp-20 activity

As in our earlier studies of LTM for spaced training (Rose et al. 2003), we used GFP to investigate whether there were pre- or postsynaptic changes in the localization or expression of tagged synaptic proteins after training. Previously, we found that 24 h after spaced training there was a decrease of GLR-1::GFP expression in the posterior ventral nerve cord region, where processes of the interneurons of the tap circuit lie, and that there was no change in the expression level of synaptobrevin/SNB-1, a vesicular protein, in the PLM mechanosensory neurons, as assayed with a P mec-7:: SNB-1::GFP marker (Rose et al. 2003). Here, we examined the same two fluorescent markers to see whether either showed changes 12 h after massed training. Because worms with a mutation in glr-1 showed normal memory 12 h after massed training, it was not surprising that there were no changes in the area of GLR-1::GFP in wild-type transgenic worms 12 h after training (data not shown). However, when we measured the area of P mec-7:: SNB-1::GFP in the synaptic terminals of the PLM mechanosensory neurons 12 h after massed training, we found that trained worms had significantly larger areas of SNB-1::GFP in this region compared to those of untrained worms (Fig. 3A, B; F1,135 = 8.18, P = 0.007), suggesting that massed training induces an increase in the number of synaptic vesicles in the presynaptic terminals of the PLM mechanosensory neurons.

To further investigate this change in SNB-1::GFP, we also tested: (1) whether the increase in SNB-1::GFP in the synaptic terminals of the PLM neurons correlated with the expression (or lack) of memory, (2) whether it was specific to massed training, and (3) whether it was dependent on flp-20. We found that the increase of SNB-1::GFP in the terminals of the PLM correlated with memory expression, as we saw no difference in the area of SNB-1::GFP in the terminals of the PLM between trained and untrained worms 24 h after massed training, a time point at which we no longer see memory (Fig. 3A, D; F12,21 = 0.27, P = NS). We also found that the increase of SNB-1::GFP in the terminals of the PLM was specific to massed training as we saw no difference in the area of SNB-1::GFP in the terminals of the PLM between trained and untrained worms 12 h after massed training (Fig. 3B, D; F12,21 = 0.51, P = NS). Finally, we found that the increase in the area of SNB-1::GFP in the terminals of the PLM was dependent upon flp-20, as we saw no difference in the area of SNB-1::GFP between trained and untrained flp-20 mutants 12 h after massed training (Fig. 3C, D; F12,171 = 0.001, P = NS).

flp-20 is required in the mechanosensory neurons for memory following massed training

Kim and Li (2004) determined that flp-20 is normally expressed in ALM, ASE, AVM, LUA, PLM, PVC, PVM, PVR, RIB/AIB, and sometimes PVT. Because we found that flp-20 is critical for the 12-h memory of massed training and that massed training induces an increase of abundance of synaptic vesicles in the terminals of the PLM mechanosensory neurons that is dependent on flp-20 activity, we hypothesized that FLP-20 peptides were required in the mechanosensory neurons (ALM, AVM, PLM, and PVM) to mediate the 12-h memory of massed training. flp-20 mutants, therefore, were transformed with a construct containing a flp-20 cDNA under the control of the mechanosensory cell-specific mec-7 promoter (Fig. 4A) and tested for their capacity to form memory 12 h after massed training. Twelve hours post-training, worms with mutations in eat-4, glr-1, or chl-1 showed evidence of memory (Fig. 2B; eat-4 and glr-1, F1,30 = 8.26, P = 0.007 and F12,33 = 5.92, P = 0.02, respectively: Fig. 2C, chl-1, F1,284 = 95.73, P < 0.0001), suggesting that the 12-h memory retention of massed training is not glutamate- or CREB-dependent.
training. Two independent lines, flp-20; yEx176 [Pmec-7::flp-20 cDNA] and flp-20; yEx177 [Pmec-7::flp-20 cDNA], showed memory 12 h after massed training (Fig. 4B; F1,42) = 4.395, P = 0.04 and F1,33 = 5.36, P = 0.03, respectively. To ensure that flp-20 activity is necessary in the mechanosensory cells, we also transformed flp-20 mutants with a flp-20 cDNA under the control of the flp-13 promoter, which drives expression in ASE, ASG, BAG, M3, M5, and DD motor neurons, but, importantly, not in the mechanosensory neurons (Fig. 4A). We again tested two independent lines (two different extra chromosomal arrays) in these experiments. As predicted, both of the transgenic lines, flp-20; yEx194 [Pflp-13::flp-20 cDNA] and flp-20; yEx196 [Pflp-13::flp-20 cDNA], did not show memory 12 h after massed training (Fig. 4C; F0,18) = 0.60, P = NS and F1,20 = 0.08, P = NS, respectively. Thus, flp-20 activity is required specifically in the mechanosensory cells for the 12-h memory of massed training.

Discussion

In this study we identified a previously unknown memory for habituation in C. elegans and have begun to elucidate a mechanism for this memory. Specifically, we found that massed training produced a 12-h memory retention of tap habituation training and that memory for massed training was mediated by a different mechanism than that for long-term memory for spaced training. Unlike long-term memory, the 12-h memory of massed training is not dependent upon protein synthesis, glutamate signaling, postsynaptic glutamate receptors, or the transcription factor CREB.

Instead, 12-h memory following massed training is correlated with a flp-20-dependent increase of synaptic vesicles in the terminals of the PLM mechanosensory neurons (as measured by an increase in the area of SNB-1::GFP), and presumably those of other mechanosensory neurons. Finally, we found that flp-20 was required specifically within the mechanosensory neurons, from which FLP-20 peptides are presumably released to activate downstream neurons to initiate the 12-h memory of massed training.

Why is there an increase in the area of SNB-1::GFP in the terminals of the PLM mechanosensory neurons after massed training? The mechanosensory neurons release glutamate and FLP-20 neuropeptides. Neuropeptides are generally released after high stimulus frequencies (for review, see Nässel 2009). Because we found that cat-4 (required for wild-type glutamate release from the mechanosensory neurons) mutants were unable to form the 12-h memory of massed training, the observed increase in SNB-1::GFP in the terminals of the PLM after massed training is unlikely to represent an increase in glutamate release. We propose that the massed training regimen induces high stimulus frequencies in the mechanosensory neurons to recruit traffic dense core vesicles containing FLP-20 neuropeptides to perisynaptic regions for release, thereby increasing the area of SNB-1::GFP in the synaptic terminals of the PLM.

Long-term memory for tap habituation in C. elegans 24 h after spaced training requires the transcription factor CREB (Timbers and Rankin 2011) and the postsynaptic AMPA-type glutamate receptor subunit glr-1 (Rose et al. 2003) in the AVA and AVD interneurons of the tap withdrawal circuit, but does not cause a change in the abundance of SNB-1::GFP in the presynaptic terminals of the mechanosensory neurons. These data suggest that the two different training protocols induce different types of memories that are mediated by distinct mechanisms. This difference between memory induced by massed and spaced training has also been found in Drosophila. In Drosophila, spaced training induces long-term memory, whereas massed training induces anesthesia-resistant memory (ARM) (Tully et al. 1994) that is similar to the 12-h memory induced by massed training in C. elegans. Like the memory induced by massed training in C. elegans, Drosophila ARM does not depend on protein synthesis or the transcription factor CREB (for review, see Margulies et al. 2005) and is mediated by presynaptic mechanisms. Increasing 5-HT release from the dorsal paired neurons onto the Kenyon cells in the mushroom bodies was found to enhance ARM, whereas decreasing 5-HT release from these neurons inhibited ARM (Lee et al. 2011). Furthermore, Knapek et al. (2011) found that the active zone protein Bruchpilot (homologous to mammalian ELKS/CAST), which functions to facilitate efficient synaptic vesicle release, was required in the Kenyon cells of the mushroom bodies for ARM. Our studies, along with those in Drosophila, highlight a fundamental difference in how the training protocol influences how memories are encoded. LTM induced by spaced training is dependent on de novo protein synthesis and CREB-dependent gene transcription, and is manifested and maintained at the level of the neural circuit/synapse predominantly by postsynaptic changes. By contrast, our study and those in Drosophila suggest that massed training results in a memory that is: (1) shorter than the memory induced by spaced training, (2) not dependent on de novo protein synthesis or on CREB-dependent gene transcription, and (3) is manifested and maintained at the level of the neural circuit/synapse predominantly by presynaptic changes, such as recruitment of vesicles containing neuropeptides that dampen the tap response. The identification of these mechanistic differences between memory induced by spaced and that by massed training contributes to our fundamental understanding of learning and how it is stored as memory.
Materials and Methods

Strains

*C. elegans* was grown at 20°C and maintained according to Brenner (1974). The following strains were obtained from the Caenorhabditis Genetics Center (University of Minnesota, Minneapolis, MN): wild-type N2 Bristol, KP4 gmr-1(m2641), MT6308 *eat-4(kky5)*, and Y117 *crh-1(z2)*, V1G126 *P-nce-7*:SNB-1::GFP was a gift from Dr. Michael Nonet (Washington University, St. Louis). The *flp-4(y35)* (Dimitriadis et al. 2010), *flp-8(pk360)*, and *flp-20(pk1596)* deletion alleles were isolated by PCR screening of populations of EMS-mutagenized strains as described (Jansen et al. 1997); double and triple mutants were generated as described (Brenner 1974). The strains NY1167 *flp-13; me279* NY1168 *flp-20(pk1596)*, ynt16x168 [Pmyo-2::GFP; Psur-5::GFP] and NY1168 *flp-20(pk1596)*; Pmyo-2::GFP; Psur-5::GFP were also used in this study (Liu et al. 2007).

Generation of transgenic animals

The *flp-20* region was amplified [FLP285 (5′-GGAAAATGGTGGCGGAGATG-3′)/FLP363 (5′-CCGACATCGTTTCGAGATG-3′)], gel purified, and microinjected (≈20 ng/μl) with the coinjection markers Pmyo-2::GFP (0.05 mg/ml) (Okkema et al. 1993) and Psur-5::GFP (0.05 mg/ml) (Gu et al. 1998), as previously described (Mello and Fire 1995). To generate the *Pmec-7::flp-20* cDNA construct, a *flp-20* cDNA was subcloned into pPD96.41 containing a mec-7 promoter. To generate the *Pflp-13::flp-20* cDNA construct, a *flp-20* cDNA was subcloned into a plasmid containing a *flp-13* promoter (pPD95.75/PFlip-13).

Heat shock treatment and behavioral testing

Habituation assays were performed, scored, and analyzed as described previously (Rose et al. 2002, 2003). To ensure that all the worms were tested at the same age, for the 24-h experiments 4-d-old worms were trained, and for the 12-h experiments 4.5-d-old worms were trained. Spaced-training trained groups were given 80 taps divided into four blocks of stimuli. Each training block consisted of 20 tap stimuli given at a 60-sec interstimulus interval (ISI) and a 1-h rest was given between blocks. For massed training, 80 taps were given in a single large block at a 60-sec ISI. Memory tests occurred 22–28 h following training for 24-h memory, and 10–16 h following training for 12-h memory. The standard protocol for these studies involved training worms in groups and then transferring worms to individual plates to be tested for memory (10 test taps at a 60-sec ISI) (Rose et al. 2002). Testing worms one at a time using this protocol took 10–12 min for each worm. In order to collect approximately 20 worms per group, it took a total of 400 min (≈6.5 h) to test trained and untrained groups for one genotype and one training paradigm. With the time we used we realized that means that one genotype would require approximately two testing days. When we had three or four genotypes we would need four or five testing days. The more testing days we have, the higher the variability (small changes in room temperature or humidity from day to day as well as age of the agar plates all affect the initial response to control; for this reason, we always ran experimental and control worms on the same day and alternated between the groups so that overall conditions were the same for groups within an experiment). As a compromise between more days/higher variability and a precise timing of tests, we chose a 4-h time window for testing 12 h after training (6 h for tests 24 h after training). The variability of the *C. elegans* tap response during memory tests between days and experiments limits the outcome of this assay to measuring the presence or absence of memory (i.e., this assay cannot reliably assess whether or not one treatment or genotype results in the retention of more memory).

For all experiments except those using *crh-1(z2)* mutants, trained and untrained control worms were tested individually. *crh-1(z2)* mutants were tested in groups of 15–20 worms on each plate as previously described (Timbers and Rankin 2011). Testing worms in the presence or absence of other worms has not been observed to alter *C. elegans* behavior during these memory tests (data not shown) and worms that are in contact with other worms during the memory test are omitted from the analysis until 1 min after their last contact with another worm.

For the relevant experiments, heat shock (32°C for 40 min) was administered as described previously (Beck and Rankin 1995; Rose et al. 2002).

Confoocal microscopy and fluorescence quantification

Confocal imaging was performed as previously (Rose et al. 2003). Briefly, GFP transgenic worm strains were mounted onto slides using 1.5 μl of 2,3-butanedione monoxime for paralysis mixed with Sephadex beads (to prevent the worms from being crushed by the coverslip [G-150–50]). In all cases, a SETCOL look-up table was used during image collection with gain and offset set to allow for imaging at an optimal intensity. The total area of GFP (microns squared) from a single stack (approximately 16–30 optical sections) was measured from the GFP clusters, where the processes of the PLM L/R neurons form chemical synapses onto the interneurons of the tap withdrawal circuit: the processes of the PLM L/R run along the ventral nerve cord for a short distance just posterior to the vulva, and there they synapse onto DVA, AVA, PDE, and AVD (White et al. 1986). In wild-type animals, this collection of about 20 synapses (determined by transmission electron microscopy) can be monitored by assaying vesicle or active zone proteins fused with a fluorescent reporter. This usually appears on confocal images as about two large synaptic varicosities (one from the PLMR and one from the PLML). Researchers blind to the treatment groups analyzed the imaging files. GFP expression was determined from collected images using NIH Image 1.63. Z projections were compiled and thresholded, and areas of GFP expression were measured as square micrometers. Final figures were generated using Adobe Photoshop 7.0.

Data analyses

Orthogonal planned comparisons using a Model I analysis of variance (ANOVA) were used to compare the reversal distances in response to tap from each experimentally trained group to those from the control untrained group of the same genotype that was run at the same time, by the same experimenter. For all statistical tests an *α* value of 0.05 was used to determine the significance. Responses to tap during the memory tests are graphically represented as percent untrained control response [(X trained − X untrained)/X untrained] × 100. The statistical analysis was performed using the statistical software Statview 4.5.

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