RESEARCH ARTICLE

Figures and figure supplements

Switch-like and persistent memory formation in individual *Drosophila larvae*

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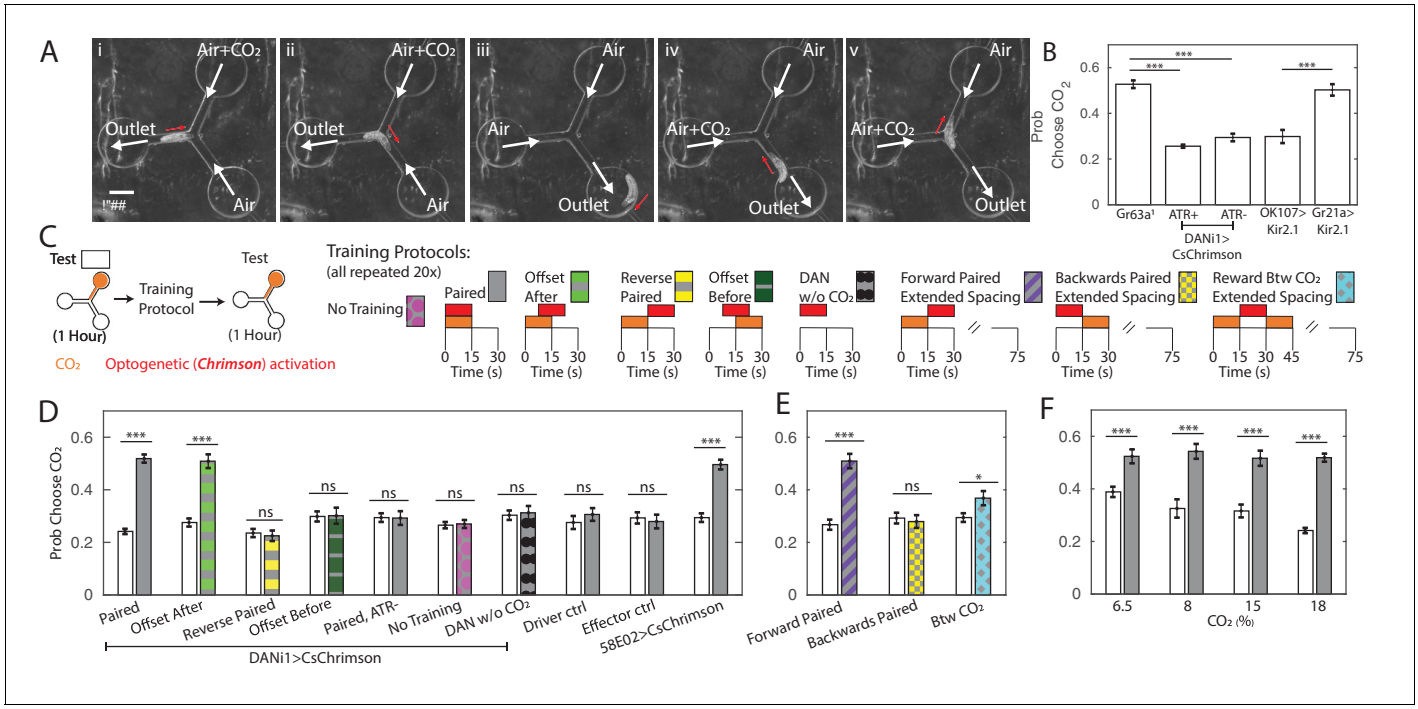

Figure 1. Y-maze assay to quantify innate and learned preference. (**A**) Image sequence of a larva making two consecutive decisions in the Y-maze assay. White arrows indicate direction of air flow; red arrow shows direction of larva's head. (**B**) Probability of choosing channel containing CO_2 without any training. (**C**) Schematic representation of experiments in (**D,E,F**). All larvae were tested in the Y-maze for 1 hr to determine initial preference and again following manipulation to determine a final preference. The manipulations were: Paired Training - reward in concert with CO_2 presentation, 15 s intervals, 20 repetitions; Offset After - reward presentation 7.5 s after CO_2 onset, 15 s intervals, 20 repetitions; Reverse-Paired Training - reward opposite CO_2 presentation, 15 s intervals, 20 repetitions; Offset Before - reward presentation 7.5 s before CO_2 onset, 15 s intervals, 20 repetitions; DAN Activation Without CO_2 - CO_2 is never presented, while reward is presented at 15 s intervals, 20 repetitions; no training - no manipulation between two testing periods; Forward Paired (extended spacing) - 15 s reward follows 15 s CO_2 presentation, followed by 60 s of air, 20 repetitions; Backwards Paired (extended spacing) - 15 s reward prior to 15 s CO_2 presentation, followed by 60 s of air, 20 repetitions; Reward Between CO_2 (extended spacing) - 15 s reward presentation between two 15 s CO_2 presentations, followed by 45 s of air, 20 repetitions. (**D**) Probability of choosing CO_2 containing channel before and after manipulation. All animals were fed ATR supplemented food, except those marked ATR-. (**E**) Probability of choosing CO_2 containing channel before and after training as a function of reward timing, in training protocols with extended air spacings. All animals were DANi1>CsChrimson and fed ATR. (**F**) Probability of choosing CO_2 containing channel before and after 20 cycles of paired training, as a function of CO_2 concentration, used both during training and testing. All animals were DANi1>CsChrimson and fed ATR. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

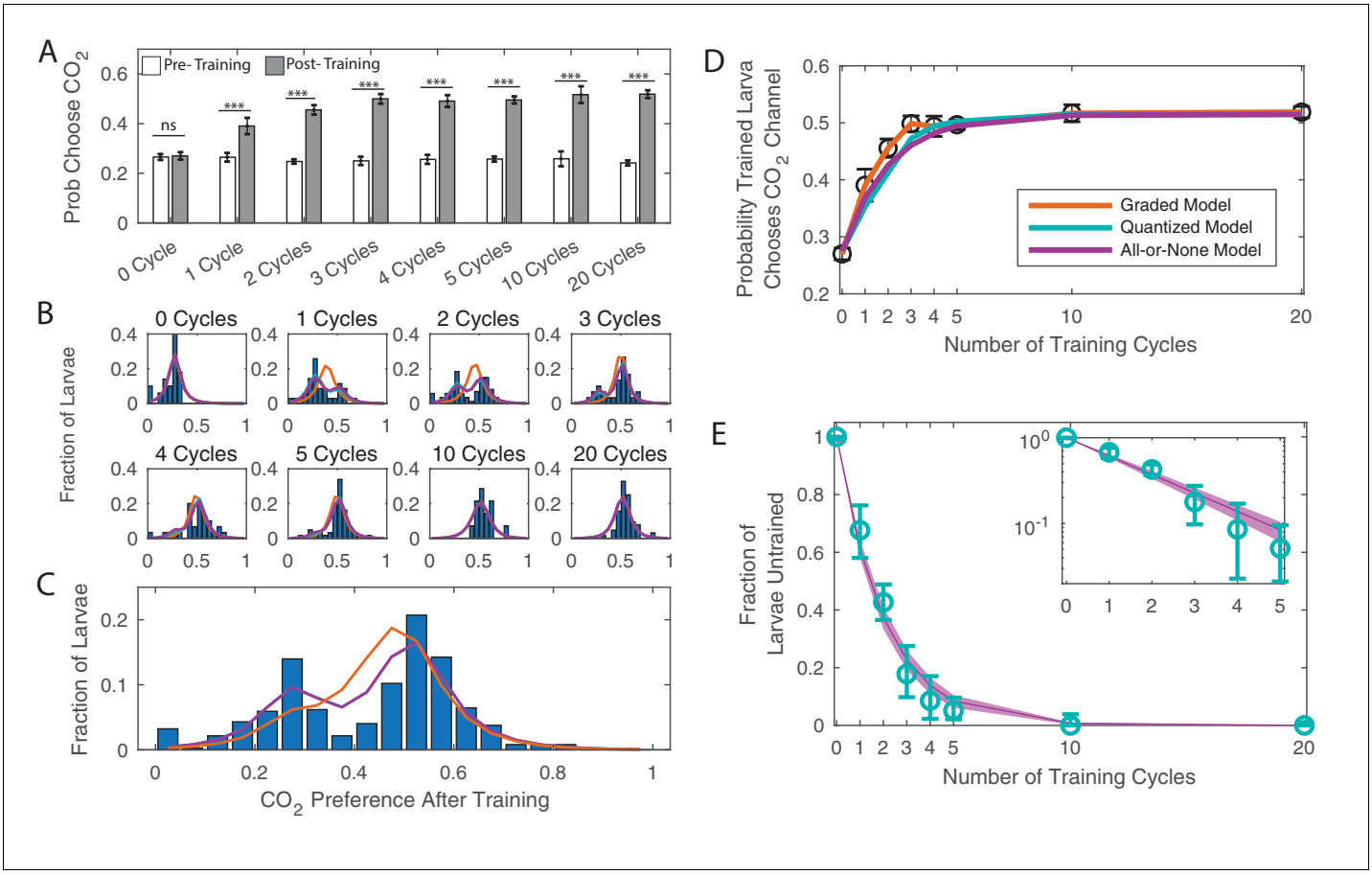

Figure 2. Dose dependence of learning DANi1>CsChrimson were given varying cycles of paired training (as in ***Figure 1C***). (**A**) Probability of choosing CO_2 containing channel before and after training, as a function of amount of training. *** p<0.001. (**B**) Histograms of individual larva preferences after training, grouped by number of training cycles. (**C**) Histogram of individual larva preference after training for all larvae. (**D**) Population average probability of choosing CO_2 following training vs. dose. (**E**) Fraction of larvae untrained vs. number of training cycles. Teal: fit parameters and error ranges from quantized model, purple lines, prediction and error ranges from memoryless model. Note logarithmic y-axis on insert. (**C–E**) Orange: graded model prediction - post-training preference is represented by a single Gaussian distribution whose mean and variance depend on amount of training; Teal: quantized model prediction - post-training preference is represented by two fixed Gaussian distributions and the fraction of larvae in each population depends on the amount of training; Purple: all-or-none model prediction - post-training preference is represented by two fixed Gaussian distributions and the effect of a single training cycle is to train a fixed fraction of the remaining untrained larvae.

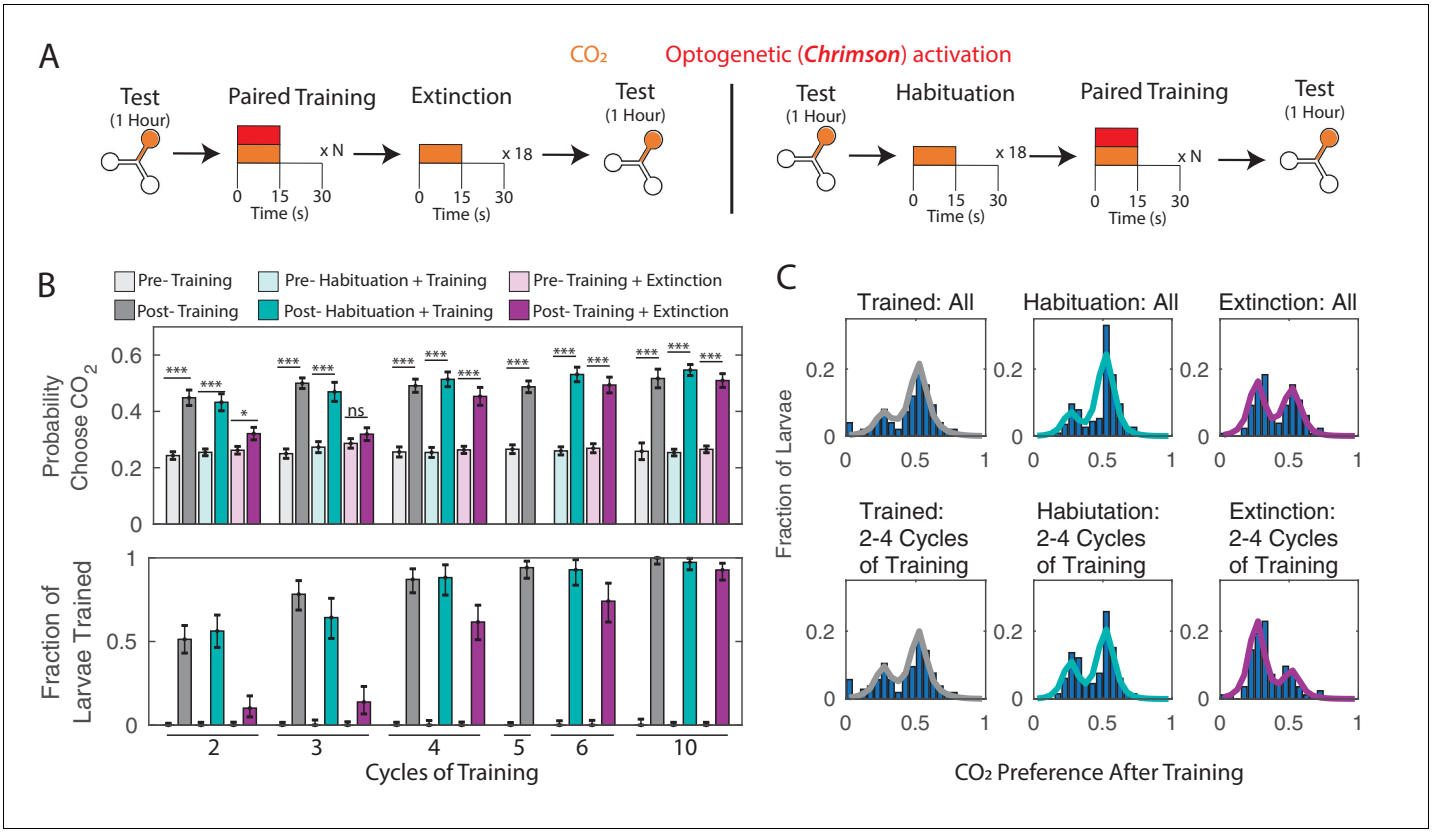

Figure 3. Memory extinction (**A**) Testing and training protocols for B,C. Training + Extinction: larvae were exposed to 18 cycles of alternating CO_2 and air following training. Habituation + Training: larvae were exposed to 18 cycles of alternating CO_2 and air prior to training. (**B**) Probability of choosing CO_2 containing channel (top) and fraction of larvae in trained group according to double Gaussian model fit (bottom) before and after training scheme. (**C**) Histograms of individual larva preference after training, for all larva and for larva trained with 2–4 training cycles. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

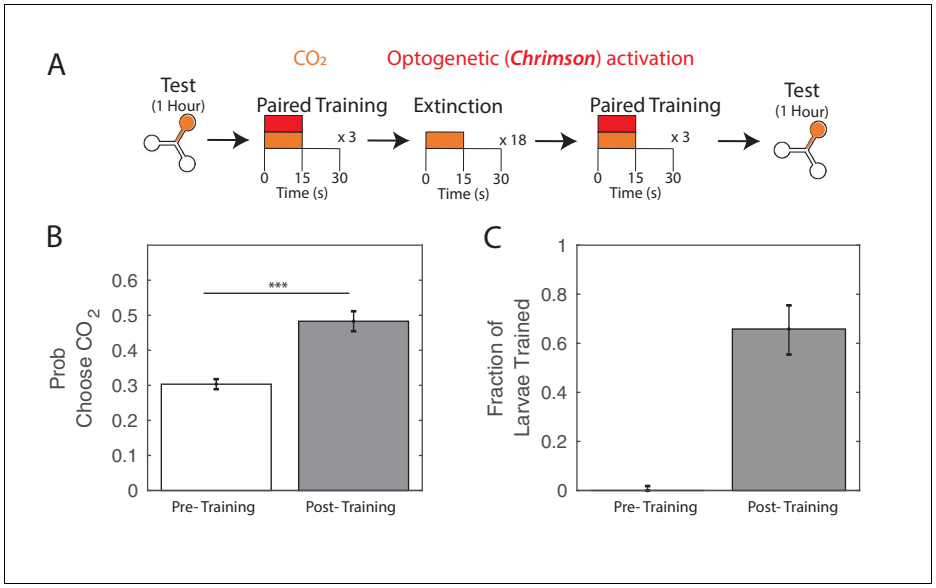

Figure 3—figure supplement 1. After extinction, larvae can be trained again. (**A**) Testing and training protocol for B,C. Larvae were trained with three cycles of paired training, followed by 18 extinction cycles of alternating CO_2 and air with no reward presented. After extinction, larvae were presented with three additional paired training cycles before testing. (**B**) Probability of choosing CO_2 containing channel before and after training scheme. (**C**) Fraction of larvae in trained group according to double Gaussian model fit before and after training scheme. All larvae were DAN-i1>CsChrimson and raised on ATR+ food. *** $p<0.001$.

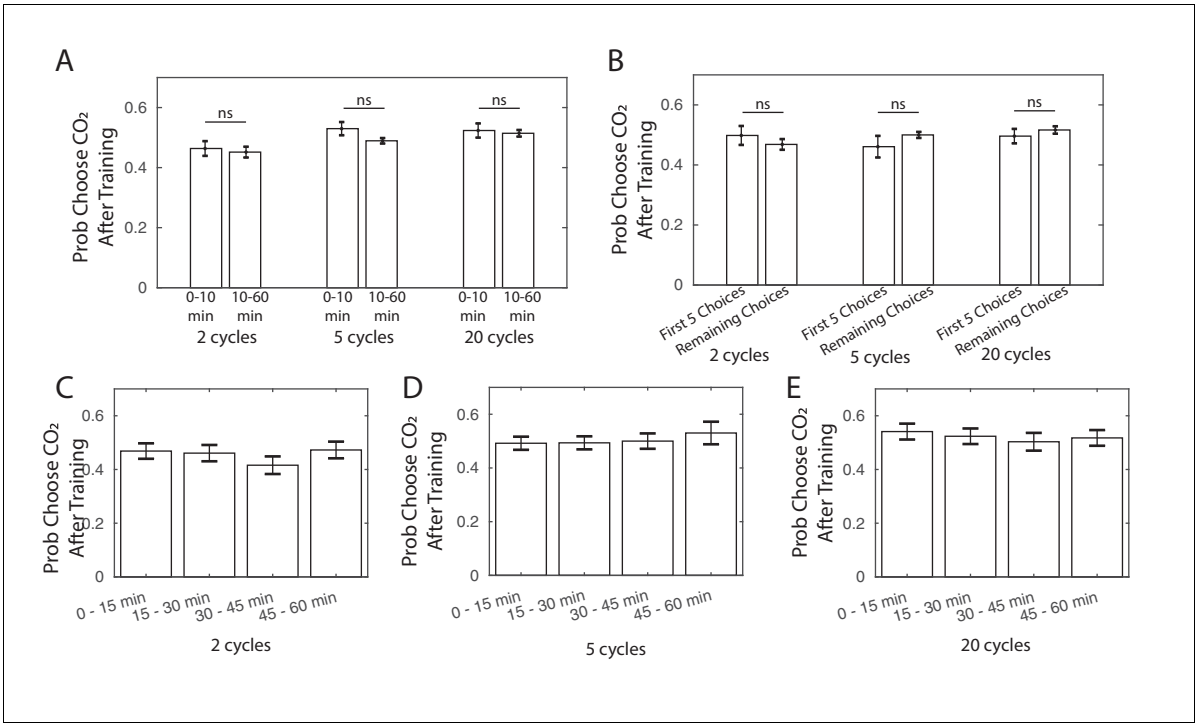

Figure 3—figure supplement 2. Larvae population average response following training. (**A**) Larvae population average response in the first ten minutes following training (0–10 min), compared to the latter fifty minutes of testing (10–60 min), for larvae that had been given 2, 5, or 20 cycles of paired training. (**B**) Larvae population average response for the first five choices made by the larvae following training, compared to the remaining choices, for larvae that had been given 2, 5, or 20 cycles of paired training and made at least 10 decisions following training. (**C,D,E**) Larvae population average response over 15-min segments following training, for larvae trained with (**C**) 2 cycles, (**D**) 5 cycles, or (**E**) 20 cycles of training. All larvae were DAN-i1>CsChrimson and raised on ATR+ food.

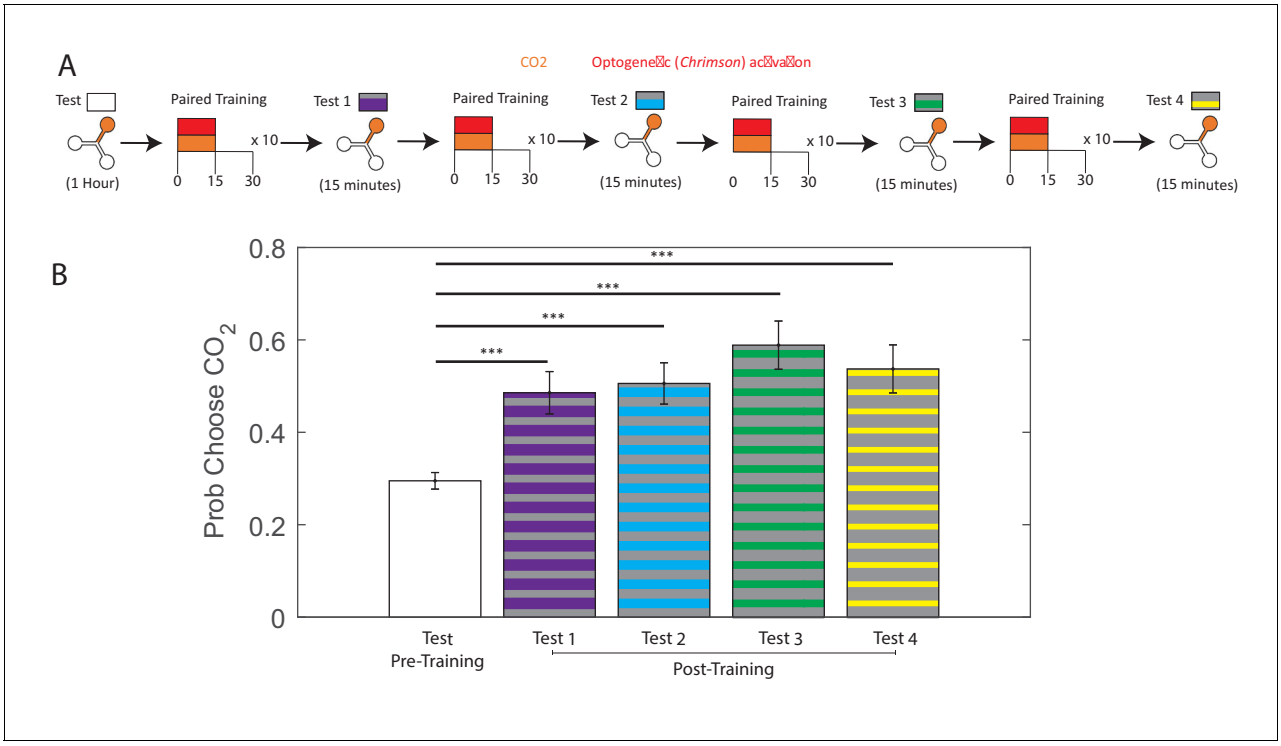

Figure 3—figure supplement 3. Larvae given additional training between testing periods. (**A**) Testing and training protocols for experiments in B. All larvae are tested in the Y-maze for one hour to determine initial preference. Larvae were then trained with 10 cycles of paired training, followed by a 15-min test period. The 10 cycle train/15 min test was repeated four times. (**B**) Probability of choosing CO_2-containing channel before training, and during each of the four test periods. All larvae were DAN-i1>CsChrimson and raised on ATR+ food. *** $p < 0.001$.

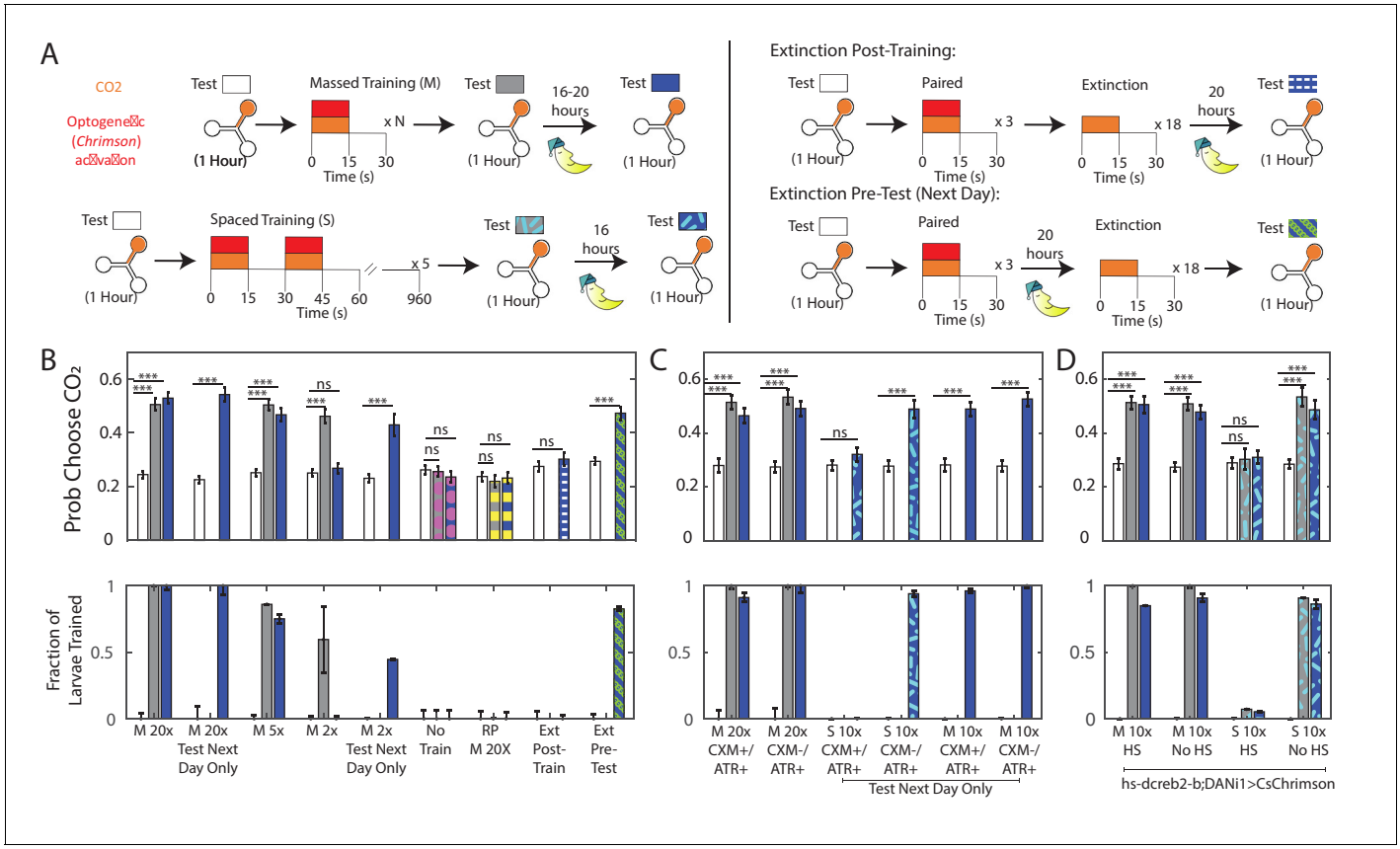

Figure 4. Memory retention overnight. (**A**) Testing and training protocols. Except where indicated, larvae were tested, trained immediately after testing, tested again, then placed on food overnight and tested the following day. For extinction experiments, larvae were trained three times, and then exposed to 18 cycles of alternating CO_2 and air either immediately following training or prior to testing the next day. (**B,C,D**) Probability of choosing CO_2 containing channel (top) and fraction of larvae in trained group according to double Gaussian model fit (bottom) prior to training, immediately following training, and the next day. When the center bar is missing, larvae were not tested immediately following training but instead removed immediately to food. M Nx = massed training, N repetitions, S 10x = spaced training 10 total pairings, RP = reverse paired (see ***Figure 1C***), No Train = no training. Larvae in (**B,C**) were DANi1>CsChrimson. Larvae in (**D**) were DANi1>hs-dCREB2-b;CsChrimson. Larvae were raised on food containing ATR, except for ATR+/CXM-, ATR+/CXM+ larvae who were fed ATR supplemented yeast paste (without/with cycloheximide) for 4 hr prior to initial testing. For reverse-paired (RP) and no training schemes, see ***Figure 1B***. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.