Supplementary data.

Ward et al.

Behavioral and Monoamine changes following severe vitamin C deficiency

Olfactory learning was included as a measure of associative learning to assess changes in cognitive ability that may occur during ascorbic acid (AA) deprivation. Training was initiated when both Gulo-/- groups were supplemented with 0.33 g/L AA. Compared to wild-type controls (WT-CON), both Gulo control (GULO-CON) and Gulo Scurvy (GULO-SCV) groups were impaired on this task during task acquisition (data provided below). The task was, therefore, excluded from the test battery that was administered during the Scurvy test period. These data are not included in the main manuscript because they do not add anything additional to the conclusion presented therein. However, they are included in supplementary data because mice were trained with the peanut butter pellets used in the pellet retrieval task, which is why these pellets were familiar to, and known to be desirable to the mice. They also present an interesting deficit in associative learning in Gulo-/- mice that has not been previously reported. It is thought that problems in Gulo-/- mice that persist through adulthood in neuromuscular ability, and now in olfactory-based associative learning, are due to AA deficiency during development in colonies in which all Gulo-/- mice are maintained on 0.33 g/L AA. In fact, this topic is under investigation in our lab, and the breeding colony is now maintained at a higher level of AA (1.0 g/L).

Methods:

Olfactory sensitivity. Olfactory sensitivity was assessed by exposing mice to 3 x 3 cm squares of filter paper soaked with water, vinegar (0.25% dissolved in water) or peanut butter (10%

dissolved in mineral oil) based on published methods (Witt et al., 2009). Following a 5-minute habituation period mice were exposed to each square for 3 minutes. The test chamber was a 40 x 30 cm clear acrylic box with a transparent, removable lid with air holes. The same chamber was used for olfactory learning and pellet retrieval. Odor order was randomized between mice and trials were videotaped and scored for time spent investigating the filter paper and number of investigations to the paper.

Olfactory learning. Olfactory learning was measured by the latency for mice to retrieve a food reward (peanut butter flavor sucrose pellet, 5TUT, 20 mg, Test Diets, Richmond, IN, USA) from a small shallow cup of scented cob bedding. The reinforced smell was either mint or lemon (dilution 1/400 in water, McCormick & Co., Inc, MD). Mice were food-deprived for 4-6 hours prior to testing and all testing took place after 13:00 hours each day. During the first session mice were given three trials following a 5 minute habituation in the test chamber. In trial 1 the pellet was placed in an empty cup. In trials 2 and 3 the pellet was placed on top of the scented cobb bedding in the cup. During the second session, for trial 1 the pellet was lightly buried in the cup with the reinforced odor. During trials 2 and 3 the pellet was lightly buried in the cup with the reinforced odor as before but this time two additional cups were added to the chamber, one with the unreinforced odor and one with no odor. For all subsequent sessions all three cups were used and their position in the chamber was varied between trials. There were two trials per session and latency to retrieve the pellet was recorded by the experimenter with a maximum trial time of 10 minutes.

Statistical Analysis. Behavioral data were analyzed with SPSS Version 19 for Mac. Repeated Measures ANOVA was used with test session as the repeated factor, and Group as the between subjects factor. Significant omnibus ANOVA was followed with Bonferroni-corrected pairwise comparisons.

Results:

Olfactory learning. One GULO-SCV mouse was excluded from the analyses because after the 2nd trial it failed to ever retrieve a pellet within the 10 min. time limit. There were significant main effects of trial with overall decreasing trial latencies across trials ($F_{(6, 120)} = 6.32$, $p<0.001$) and also of group ($F_{(2, 20)} = 5.48$, $p<0.05$), but no interaction between the two factors ($F_{(12, 120)} = 0.73$, $p=0.72$). WT-CON mice were consistently quicker to retrieve the pellets than either GULO-CON or GULO-SCV groups (ps<0.05, Fig. S1). Gulo-/- groups did not differ across trials (p=1.0).

Olfactory sensitivity. Videos were unavailable for coding from two peanut butter trials (GULO-CON; GULO-SCV) and one vinegar trial (GULO-SCV). There were no differences among the groups on either time spent smelling the three odors, or number of visits to the scented filter paper (Water: Time $F_{(2, 21)} = 1.86$, $p=0.18$, Visits $F_{(2, 20)} = 0.72$, $p=0.50$; Peanut butter: Time $F_{(2, 19)} = 1.65$, $p=0.22$, Visits $F_{(2, 19)} = 2.44$, $p=0.11$; Vinegar: Time $F_{(2, 20)} = 0.30$, $p=0.74$, Visits $F_{(2, 20)} = 1.41$, $p=0.27$; *data not shown*).

Cited reference

Witt RM, Galligan MM, Despinoy JR, Segal R (2009) Olfactory behavioral testing in the adult mouse. Journal of visualized experiments : JoVE.

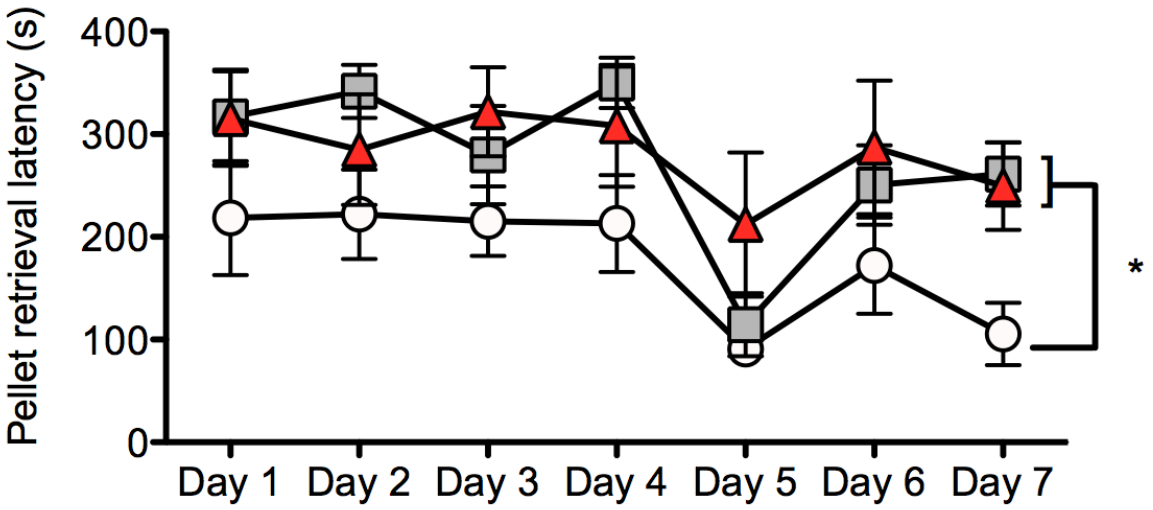

Figure S1. Time taken to retrieve pellets during olfactory learning acquisition training.

There was an overall difference across testing sessions between WT-CON (white circles) and both GULO-CON (grey squares) and GULO-SCV (red triangles), *$p<0.05$. It should be noted that at the time of training (Days 1-6), all Gulo mice were maintained on the same 0.33 g/L AA supplements. Day 7 of training occurred following 2 weeks of AA deprivation in the GULO-SCV mice during which time AA levels would be decreasing, but would not be low yet.