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Effect of vitamin C deficiency during postnatal development on adult behavior: functional phenotype of *Gulo*($-/-$) knockout mice

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Abstract

Organisms using oxygen for aerobic respiration require antioxidants to balance the production of reaction oxygen species during metabolic processes. Various species — including humans and other primates—suffer mutations in the *GULO* gene encoding *L*-gulono- γ -lactone oxidase; *GULO* is the rate-limiting enzyme in the biosynthesis of ascorbate, an important cellular antioxidant. Animals lacking the ability to synthesize vitamin C develop scurvy without dietary supplementation. The *Gulo*($-/-$) knockout mouse requires oral supplemental vitamin C; without this supplementation the animal dies with a scorbutic condition within several weeks. Vitamin C is known to be most abundant in the brain, where it is believed to play important roles in neuroprotection, neurotransmission, and neuromodulation. We therefore hypothesized that ascorbate deficiency in *Gulo*($-/-$) knockout mice might lead to an abnormal behavioral phenotype. We established the amount of ascorbate in the drinking water (220 ppm) necessary for generating a chronic low-ascorbate status in the brain, yet clinically the mice appeared healthy throughout 100 days postpartum at which time all behavioral-phenotyping tests were completed. Compared with *Gulo*($+/+$) wild-type littermates, ascorbate-deficient *Gulo*($-/-$) mice were found to be less active in moving in their environment; when in water, these mice swam more slowly in some tests, consistent with a mild motor deficit. We found no evidence of cognitive, anxiety, or sensorimotor-gating problems. Despite being less active, *Gulo*($-/-$) mice exhibited exaggerated hyperactivity to the dopaminergic agonist methamphetamine. The subnormal movement, combined with hypersensitivity to a dopamine agonist, point to developmental ascorbate deficiency causing long-term striatal dysfunction.

Keywords

acoustic startle response; ascorbic acid; elevated zero maze; *L*-gulono- γ -lactone oxidase; locomotor activity; methamphetamine challenge; Morris water maze; novel object recognition; oxidative stress in brain; vitamin C

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Introduction

Most multicellular organisms require atmospheric oxygen for their existence, yet oxygen in the cell is also transformed to reactive intermediates responsible for mutations, cancer, aging, and other diseases (Jones 2006). Defending against reactive oxygen species (**ROS**), organisms possess “oxidative stress response” systems that function to prevent oxidative damage to cellular macromolecules (Jones 2006; Hur and Gray 2011; Runchel *et al.* 2011). ROS have some useful functions: participation in redox signaling (Packer and Cadenas 2011) and protection against autoimmune disease (Hultqvist *et al.* 2009); thus, the role of antioxidant systems is not to remove ROS entirely, but to maintain a balance of ROS levels (Jones 2006; Rhee 2006).

Prominent antioxidants include: ascorbic acid (**vitamin C**) (Harrison and May 2009), reduced glutathione (**GSH**) (Dalton *et al.* 2004), lipoic acid (De Araujo *et al.* 2011), uric acid (Amaro *et al.* 2008), carotenes (Obulesu *et al.* 2011), tocopherols and tocotrienols (**vitamin E**) (Clarke *et al.* 2008), ubiquinone and ubiquinol (**coenzyme Q**) (Dhanasekaran and Ren 2005), and melatonin (Reiter *et al.* 2010).

Ascorbate is a strong electron donor. All biological functions of ascorbate can be attributed to its reducing properties, including the scavenging of electrophiles and serving as enzyme co-factors in biosynthesis (Englard and Seifter 1986). Ascorbate is most abundant in brain, suggesting a significant role in central nervous system (**CNS**) function (Hornig 1975), but biosynthesis in mammals occurs in liver; ascorbate accumulation in brain occurs via ascorbic acid uptake by SLC23A2 transporters in the choroid plexus (Tsukaguchi *et al.* 1999). Ascorbate has several roles in the CNS (Harrison and May 2009). First, it acts as a neuroprotective agent against ROS. Second, it regulates dopamine- and glutamate-mediated neurotransmission by serving as a cofactor in converting dopamine to norepinephrine and modulating glutamate transmission. Third, it is involved in neuronal maturation. In rodents, ascorbate levels are highest in developing brain in the early postnatal period (Terpstra *et al.* 2010), and expression of SLC23A2 peaks shortly after weaning (Meredith *et al.* 2011).

Ascorbic acid deficiency leads to scurvy. Some mammals, including humans, cannot synthesize vitamin C due to mutations in the *GULO* gene encoding *L*-gulono- γ -lactone oxidase (**GULO**), the rate-limiting enzyme in ascorbate biosynthesis (Nishikimi *et al.* 1994). The *Gulo*($-/-$) knockout mouse mimics this condition (Maeda *et al.* 2000). Although scurvy is rare in children in developed countries, subclinical hypovitaminosis C is common in many parts of the world during pregnancy and in young children. Such deficiency has unknown long-term consequences on brain development and behavior (Tveden-Nyborg and Lykkesfeldt 2009), but obvious adverse effects on the developing guinea pig brain (Tveden-Nyborg *et al.* 2009). Despite these concerns, there are few studies that have attempted to address the effect of vitamin C deficiency on brain functions (Oria *et al.* 2003; Harrison *et al.* 2008). The purpose of this experiment was to phenotype *Gulo*($-/-$) compared with *Gulo*($+/-$) mice using a wide range of established behavioral tests (Vorhees and Williams 2006; Curran *et al.* 2011).

Materials and Methods

Experimental subjects and diet

Generation of the original mouse line carrying the disrupted *Gulo* gene has been described (Maeda *et al.* 2000). *Gulo*($+/-$) breeders on a >99.8% C57BL/6J background were purchased from The Jackson Laboratory (Bar Harbor, ME) and have been maintained in our mouse colony for ~7 years. *Gulo*($+/-$) heterozygotes were bred in order to generate *Gulo*($+/-$) wild-type (**WT**) controls and *Gulo*($-/-$) knockout (**KO**) offspring for all experiments

reported herein. Because standard lab chow can contain varying amounts of vitamin C, all mice were continuously maintained on ascorbate-free Teklad AIN-93G Purified Diet. The heterozygous parents require no vitamin C supplementation. Because of transfer of ascorbic acid from mother to offspring in utero and during lactation, *Gulo*($-/-$) pups were not given vitamin C supplementation until weaning at postnatal day (PND) 21. Drinking water was supplemented with *L*-ascorbic acid (220 ppm) from PND21 through PND100 (end of the behavioral-testing period) for both KO and WT offspring. Freshly-prepared ascorbate-containing water was administered twice a week. Animals were housed in an AAALAC-accredited vivarium; the animals were treated humanely and in accordance with the NIH Guide for the Care and Use of Laboratory Animals and the protocol was approved by both the University of Cincinnati and Cincinnati Children's Research Foundation Institutional Animal Care and Use Committees.

Breeding

Nulliparous females (20–25 g body weight) aged 3–5 months were used for all matings. The morning when a vaginal plug was found was considered gestational day (GD)0.5; plug-positive females were removed from the breeding cages. Pregnant females were housed individually with pups until weaning on PND21.

Assays for ascorbate and GSH levels in brain

Because we previously had found no sex differences in ascorbate or GSH levels, brain tissue was collected only from males (N=4 for each time-point). All mice were maintained on the AIN-93G diet and supplemented with 220 ppm ascorbic acid at weaning. Brains were dissected on PND21 or PND60 and stored at -80°C until the assays were performed. Ascorbate levels in whole brain homogenates were determined by spectrophotometric measurement of ferritin iron released by ascorbate as described (Zannoni *et al.* 1974). GSH levels in whole brain homogenates were measured fluorometrically after α -phthalaldehyde derivatization, as described (Senft *et al.* 2000).

Behavior

This experiment used a behavioral-test battery, as has been recommended for phenotyping of genetic and other models having CNS effects (Vorhees 1996; Vorhees 1997; Crawley 1999; Crawley 2000; MacQueen *et al.* 2001; Branchi *et al.* 2003; Bailey *et al.* 2006; Paylor *et al.* 2006; Crawley 2007). Methods were chosen to include tests of anxiety, exploration and habituation, sensorimotor gating, and two tests of learning and memory—one using aversive motivation and one using spontaneous recognition of familiarity.

Animals from both genotypes were tested in groups. One male and one female per litter per genotype were tested (N=18–27/group) beginning at PND60. Week-1: elevated zero maze (EZM), locomotor activity, acoustic startle response (ASR) with prepulse inhibition (PPI). Week-2: novel-object recognition. Week-3: Morris water maze (MWM) cued. Week-4: MWM hidden acquisition. Week-5: MWM hidden reversal. Week-6: MWM hidden shift. Week-7: locomotion with (+)-methamphetamine (1 mg/kg) challenge. All tests were performed during the light portion of the 14:10 light:dark cycle

EZM

The apparatus that we used was a circular runway (105-cm diameter), 72 cm above the floor, with a 10-cm path divided in equal quadrants. Two quadrants opposite one another have 28-cm walls and two have 1.3-cm acrylic curbs. Mice were videotaped for 5 min. Time-in-open, latency-to-first-open-zone entry, head-dips, and zone-crossings were scored (Shepherd *et al.*, 1994).

Locomotor activity

The clear acrylic test arenas measured 40×40 cm (16 infrared LED-photodetectors arrayed in the x- and y-planes and spaced at 2.5-cm intervals); mice were tested for 1 h with data recorded at 5-min intervals (Accuscan Instruments; Columbus, OH).

ASR-PPI

An SR-LAB apparatus (San Diego Instruments; San Diego, CA) was used with 5-min acclimation, followed by a 4×4 Latin square of four trial types repeated three times: no-stimulus, startle signal (SS), 74-dB prepulse + SS, or 76-dB prepulse + SS. Inter-trial interval was 8 s and the inter-stimulus interval was 70 ms. Signal was a mixed-frequency white noise burst (120 dB SPL, for 20 ms). Peak response amplitudes ($V_{\max}$), recorded during a 100-ms window following SS onset, were analyzed.

Novel object recognition

Mice were tested in open arenas in a room with many distal cues available, using a spatial novel object method (Clark *et al.* 2000) modified for mice. Mice were first habituated to arenas (91-cm diameter) for 2 days, followed by 2 days of exposure to two objects (10 min/day) to prevent neophobia during the test phase. On the final day, new but identical objects were presented until 30 s of cumulative observation of the objects was accrued; 1 h later, the familiar (an exact copy) and a novel object were presented until 30 s of cumulative observation was accrued (up to 10 min). Object types for the test were counterbalanced to prevent possible bias from inherent preferences for one object over the other.

MWM

The tank was 122 cm in diameter, 50 cm in height, and filled to a depth of 25 cm (Vorhees and Williams 2006). Day-1: six cued trials were given with the start and the platform fixed (curtains were closed to obscure distant visual cues). Days 2–6: two trials per day were given with random start and goal positions (curtains remained closed); the 10-cm platform contained an orange ball mounted 10 cm above the surface to clearly mark its location.

Mice received three phases of hidden-platform testing (curtains open), four trials per day for 6 days with a 30-s probe trial on Day-7. Each phase used a different platform size (acquisition = 10, reversal = 7, and shift = 5 cm in diameter). During acquisition, the platform was placed in the SW quadrant; during reversal it was in the NE quadrant; and during shift it was in the NW quadrant, using the position of the experimenter as the S position (not actual compass directions). The platform was positioned within each quadrant halfway between the center and wall of the pool. Data were analyzed for latency, cumulative distance, path length, and swim-speed for platform trials and on probe trials for number of platform site crossovers, average distance to the platform site, and target quadrant preference.

Locomotor activity with drug challenge

In order to reacclimatize the mice to the test chamber prior to drug challenge, mice were placed in the locomotor-activity-test chambers and allowed to habituate. After 30 min, the test was stopped and each mouse was removed, injected subcutaneously with 1.0 mg/kg (+)-methamphetamine HCl (expressed as the free base), and returned to the test chamber; the monitoring was then continued for an additional 120 min.

Statistical analyses

Behavioral data were analyzed using mixed-linear analysis of variance (ANOVA) with repeated-measures, or by analysis of covariance (ANCOVA) (SAS version 9.2, SAS

Institute; Cary, NC). Results were considered statistically significant if $P < 0.05$. Follow-up analyses were done by slice-effect ANOVAs for significant interactions. Because there were only two groups, pairwise-group comparisons were performed by Student two-tailed t-test for independent samples.

Results

Ascorbate supplementation

Regular lab chow contains vitamin C (~110 mg/kg), which can vary considerably from lot to lot; in any event, this dose is unable to support normal growth of post-weanling *Gulo*($-/-$) mice. Without proper amounts of dietary vitamin C, plasma and tissue ascorbic acid levels within 2 weeks are ~15% of normal and by 5 weeks *Gulo*($-/-$) mice lose weight, become anemic, and die; plasma total antioxidant capacities are ~37% of that in *Gulo*($+/-$) heterozygotes and *Gulo*($+/+$) wild-type mice (Maeda *et al.* 2000). In the present study, *Gulo*($+/-$) dams received an ascorbate-free diet throughout the gestation and lactation stages. Despite this ascorbate-free diet, endogenously-synthesized vitamin C can be transferred from dam to the offspring via the placenta and milk. Indeed, we found that brain ascorbate levels in KO weanlings (PND21) were ~30% of that in WT pups (Fig. 1A). This level of ascorbate deficiency in brain from pre-weanling KO animals is in agreement with an earlier study (Harrison *et al.* 2010).

We therefore titrated ascorbic acid supplementation in the drinking water—searching for a proper amount of vitamin C to sustain a comparable level of ascorbate deficiency in post-weanling KO mice prior to and throughout the PND60-PND100 period of behavioral testing.

Ascorbate and GSH levels in brain

Fig. 1A shows that, with a dose of 220 ppm in the drinking water starting at PND21, brain ascorbate levels in KO mice remain about ~25% of normal at PND60, indicating a status of chronic ascorbate deficiency from early postnatal development until behavioral testing began. Given the interplay between the two antioxidants ascorbate and GSH (Meister 1994), we also measured brain GSH levels at PND21 and PND60 (Fig. 1B). Interestingly, brain GSH levels in weanling KO mice at PND21 were up-regulated, which has been reported in the earlier study (Harrison *et al.* 2010). This apparent compensatory effect disappeared at PND60.

Body weight

Gulo($+/+$) and *Gulo*($-/-$) offspring were weighed weekly and did not show significant body-weight differences within each sex group. At PND100, the means and S.E.M. of male WT and KO were 24.8 ± 0.54 g (N=20) and 24.3 ± 0.58 g (N=17), respectively, and female WT and KO weighed 19.3 ± 0.60 g (N=16) and 20.0 ± 0.47 g (N=26), respectively. KO mice appeared outwardly as healthy as WT during post-weaning and the entire period of behavioral phenotyping—suggesting that KO mice receiving ascorbate at 220 ppm in the drinking water can be rescued from scurvy and other severe effects of ascorbate deficiency.

EZM

We found no significant effects of genotype (data not shown) on any measure of EZM performance (time-in-open, zone crossings, latency-to-first-open-zone entry, or head-dips over the edge). Also, we saw no genotype $\times$ sex interactions for time-in-open, latency, or head-dips; however, there was a significant interaction on zone-crossings ($F_{1,59} = 6.98$, $P < 0.01$). Slice-effect ANOVAs on each sex showed no genotype effect in males, but a significant genotype effect for females ($P < 0.02$); the means $\pm$ S.E.M. for females were WT = 4.3 ± 0.6 and KO = 6.3 ± 0.5 entries). Hence, there was no evidence of changes in anxiety-

like behavior (time-in-open, latency-to-first-open-zone entry, or head-dips), but female KO mice crossed more open zones—suggesting increased activity in a novel environment. Although not significant, male KO mice showed the opposite pattern (WT = 5.1 ± 0.6 and KO = 3.7 ± 0.8 entries, $P < 0.17$).

Locomotor activity

A significant genotype main effect was observed on locomotor activity ($F(1,83.2) = 4.57$, $P < 0.05$; Fig. 2). There were no genotype interactions with sex, interval, or the combinations thereof. As seen in Fig. 2, KO mice were significantly less active than WT, throughout the 60-min test session.

ASR with PPI

There were no significant effects of genotype or interaction of genotype with other factors (sex or trial) on ASR amplitude, with or without prepulse, or at any of the prepulse intensities tested (data not shown). Hence, KO mice showed no evidence of the sensorimotor-gating problems that are often associated with major psychiatric disorders such as schizophrenia, bipolar disorder, or major depression.

Novel-object recognition

During the familiarization trial, KO and WT mice showed no significant differences in their preference for either of the training objects. On the novelty-test trial given 1 h later, there was a significant effect of genotype on novel-object preference ($F(1,71) = 4.22$, $P < 0.05$). Compared with WT mice (mean percent novel preference $\pm$ SEM = $63.6 \pm 1.8\%$), KO mice showed significantly increased preference for the novel object over the familiar object ($68.9 \pm 1.8\%$), suggesting that KO mice might have enhanced memory (See Fig. 2 for groups sizes). Spatial object memory is associated with dorsal hippocampal function (Clark *et al.* 2000).

MWM, cued platform

Day-1 involved six trials with the same start and goal positions; the purpose was to train mice that the platform was the goal and the only means of escape from the water—if they climbed and remained on the platform until being removed by the investigator. No genotype effect was found, but there was a genotype $\times$ trial interaction ($F(5,317) = 2.67$, $P < 0.05$). Follow-up slice-effect ANOVAs on each trial showed only one effect (Fig. 3, *left*), and this occurred on Trial-6 ($P < 0.05$); on this trial, KO mice reached the platform significantly more quickly than WT. Because performance on these trials is not dependent on memory, this slight improvement in performance on trial-6 in the KO mice may not be meaningful.

Days 2–6 involved two trials per day, but with the start and goal randomly positioned; the purpose was to train mice to search for the platform using only the platform as a direct cue, since the surrounding room cues were obscured by curtains. Figure 3 (*right*) shows that there was a genotype $\times$ sex interaction ($F(1,80.3) = 9.72$, $P < 0.01$). Slice-effect ANOVAs on female performance showed a trend for shorter latency ($P = 0.07$), while a significant effect was found for males ($P < 0.05$). KO males took significantly longer to reach the goal than WT males. Because these trials do not require memory but only that the mouse sees the platform in order to find it, the fact that KO males took longer to reach the platform suggests a slight motor impairment in their ability to swim.

MWM hidden platform

There were no genotype main effects or interactions with genotype on indices of associative learning (latency, path-length, and cumulative distance) to find the hidden platform during

acquisition (phase-1) or shift (phase-3) testing (Fig. 4), *i.e.* all groups showed typical learning curves as a function of phase difficulty. The path length is presented in Fig. 5 because it is not affected by swim speed. There was no genotype main effect on reversal (phase-2), but a significant genotype $\times$ sex $\times$ day effect was seen ($F(5,288) = 2.52, P < 0.05$). Slice-effect ANOVAs revealed that on Day-2 there was a significant genotype effect (females, $P < 0.02$) and on Day-3 there was a trend in males ($P < 0.07$) (not shown). The interaction of genotype $\times$ sex $\times$ day was also significant for cumulative distance from the platform on reversal ($F(5,281) = 2.37, P < 0.05$). However, in this case, slice-effect ANOVAs showed the effect to be significant on Days 2–3 only in males. This inconsistency between the interaction effects on path length versus cumulative distance suggests that the effect is not reliable or meaningful.

For swim speed, there was no genotype main effect during acquisition but there was a trend ($F(1,83.4) = 3.73, P < 0.06$), and there was a significant genotype main effect during reversal ($F(1,75.2) = 10.42, P < 0.01$) (Fig. 5A). No interactions with genotype were observed. During the shift phase (Fig. 5B) there was also a significant swim-speed main effect ($F(1,83.1) = 17.62, P < 0.0001$) and genotype $\times$ sex interaction ($F(1,83.1) = 7.94, P < 0.01$). Slice-effect ANOVAs on each sex showed no significant genotype effect among females, but a significant effect among males ($P < 0.0001$). The means $\pm$ S.E.M. for males were KO = 18.1 ± 0.5 and WT = 21.2 ± 0.4 cm/s. These data are consistent with the locomotor-activity data and MWM-cued data. In other words, male KO mice are slower and less active than WT, indicating a reduced vigor on some tests, while providing no evidence of a cognitive deficit.

On the probe trials given 24 h after the last training day of each phase, no genotype or genotype $\times$ sex interaction was found on average distance to the platform site, crossovers, or percent time or distance in the target quadrant for acquisition, reversal or shift probe; however, there were effects on swim speed on some probe trials (Fig. 6). There were no significant swim-speed effects on acquisition-probe, but significant swim-speed effects were seen on the reversal and the shift probe. On reversal-probe (Fig. 6A), there was both a genotype main effect ($F(1,75) = 4.74, P < 0.05$) and a genotype $\times$ sex interaction ($F(1,75) = 5.04, P < 0.05$). On shift (Fig. 6B), there was no genotype main effect, whereas a genotype $\times$ sex interaction was found ($F(1,83) = 4.60, P < 0.05$). Both of the significant sex interaction effects in KO mice are shown in Fig. 6. In both cases, male KO mice swam more slowly, a finding seen in several of the preceding indices and confirming a general sluggishness in male KO mice under some circumstances.

Locomotor activity with drug challenge

During the 30-min rehabilitation phase, there was no significant main effect of genotype or genotype $\times$ interval or genotype $\times$ sex interactions; however (Fig. 7, *left*), there was a significant three-way interaction of genotype $\times$ sex $\times$ interval interaction ($F(5,282) = 2.38, P < 0.05$). Slice-effect ANOVAs on each interval for each sex showed no significant genotype effect at any of the pre-challenge intervals, but one interval did show a trend in the first 5 min of the test in males ($P < 0.07$), during which time KO males were slightly less active than WT males (means $\pm$ S.E.M.: WT = $1,405 \pm 62.5$, KO = $1,228 \pm 70.6$ beam interruptions).

In order to control for even minor starting differences when analyzing the post-challenge data, we analyzed the post-challenge data using ANCOVA; we used the mean of the last two pre-challenge intervals as the covariate in a repeated-measure ANCOVA. This analysis showed a significant main effect of genotype ($F(1,75.6) = 7.09, P < 0.01$) and a significant genotype $\times$ interval interaction ($F(23,1591) = 1.66, P < 0.05$). Slice-effect ANOVAs, at each interval for genotype, showed significant differences on post-challenge time intervals 15–70

and 85 min (Fig. 7, *right*). At each of these intervals, KO mice were significantly more active than WT.

Discussion

The *Gulo*($-/-$) knockout mouse line was created in order to further investigate the effects of genetically-modified ascorbate synthesis deficiency (Maeda *et al.* 2000). In the present experiments, we induced a subclinical level of hypovitaminosis C during postnatal brain development and early adulthood, in order to model the potential of this more widespread condition to affect long-term outcomes. We found clear phenotypic differences, which are in general agreement with other models of vitamin C deficiency (Oria *et al.* 2003; Harrison *et al.* 2008).

We found that *Gulo*($-/-$) mice, supplemented with low levels of ascorbate throughout development and early adulthood, showed multiple phenotypic differences, compared with WT mice. These changes in the KO mice included: an increase in zone crossings in the EZM in females, but no change in the indices of anxiety; a relatively uniform reduction in locomotor activity in an automated open-field throughout the 1-h test (both sexes); increased new object preference in novel object recognition (both sexes); slower swimming on cued trials with a randomly placed goal (males only), but not on trials with a fixed goal (except on the last trial where KO males and females were faster); reduced swim speed during hidden platform testing on acquisition (both sexes, trend), reversal (both sexes) and shift (males only), and on reversal probe (males only) and shift probe (males only) trials in the MWM but not on acquisition probe; and finally, no change during habituation in the automated open-field but exaggerated hyperactivity after being challenged with methamphetamine (both sexes).

This complex pattern, with more effects in males than in females, may reflect a slight motor dysfunction—given that path length and cumulative distance indices in the MWM showed no genotype differences and no index of spatial memory impairment was observed on probe trials of retention. In light of this pattern, the increased novel object preference in the *Gulo*($-/-$) mice may reflect either increased memory for the familiar object or a change in attention or curiosity for novelty; however, the latter is not consistent with the open-field or EZM data, because increased novelty preference would be expected to increase open-field exploration and/or central zone activity, and increase time in open quadrants in the EZM, neither of which occurred. Alternatively, if the increased novel object preference was not attributable to increased memory, then no change in MWM spatial memory would be expected, and this is consistent with the absence of retention deficits on probe trials at the end of all three phases of MWM testing. Without further experiments it is not possible to determine whether the NOR change is related to memory or other factors.

The reduced swim speed in the *Gulo*($-/-$) mice, seen primarily on the cued random platform trials and the hidden platform reversal and shift trials (predominately in males), may reflect increased frustration because these are the most difficult tasks. It is possible that the KO mice were uncertain which way to swim between the previously reinforced platform location and the new one. Deficits in reversal and shift learning are reliably seen in rodents with hippocampal injury. However, if this were the case in *Gulo*($-/-$) mice, one would have expected to see increased path lengths in the MWM and impaired recognition on NOR tests; neither of which were observed.

An alternative explanation may be that KO mice have an altered emotional response to the more difficult phases of the MWM and thus are less able to adapt to the changed reinforcement contingency created by moving the platform to a new location. This might

cause the KO mice to be hesitant in deciding which direction to swim. A third possibility is that *Gulo*($-/-$) mice may have greater retrograde interference during reversal and shift; however, if this were the case, their response is not typical of what would be predicted by this effect. More typically, retrograde interference results in longer path lengths and higher cumulative distances, because the animals return to their previously learned location first; only when they cannot find it, do they swim to the new location. However, it cannot be ruled out that retrograde interference could cause a delayed response rather than perseveration. To determine which of the above explanations best accounts for the MWM speed differences, future experiments will need to be designed to specifically test each of these alternative hypotheses.

Previous behavioral studies in vitamin C-deficient rodents

Guinea pigs have a mutation in the *Gulo* gene and therefore are naturally vitamin C-deficient. After feeding guinea pigs vitamin C-deficient chow, an intra-gastric dose of vitamin C (100 vs 5 vs 0 mg per day) was administered to the animals for 26 days—which is sufficient time for scurvy to develop in the unsupplemented group. Differences found in the scorbutic group included: weight loss, decreased size of the periodontal ligament, abnormal spinal cord morphology involving lower motor neurons, atrophy of the gastrocnemius muscle, and deficiencies in running, climbing, and rearing (Oria *et al.* 2003). The latter deficiencies are partially consistent with observations in the present study of diminished motor activity and slower swimming on some tests seen in the *Gulo*($-/-$) mice.

By contrast, another guinea pig study put one group on a vitamin C-deficient diet and another on a standard diet during development. These investigators found no differences in the MWM on learning trials, but reduced performance on reference memory in the vitamin C-deficient group—using a delayed probe trial approach in which the probe trial was given 4 days after the last training trial (Tveden-Nyborg *et al.* 2009). We did not use a delayed probe trial in the present study; therefore, it is unknown whether *Gulo*($-/-$) mice have more rapid forgetting than WT controls, but this could be tested in future experiments.

Utilizing a different set of behavioral tests, Harrison and coworkers found that young adult *Gulo*($-/-$) KO mice, rendered ascorbate-low via acute deprivation of oral ascorbate, were cognitively normal, but showed a defect in strength and agility (Harrison *et al.* 2008). Comparing *Gulo*($+/+$) WT with *Gulo*($-/-$) “sufficient” (0.33 g/L ascorbate in drinking water) with *Gulo*($-/-$) “low” (0.033 g/L in drinking water) mice, they found F₄-neuroprostanes (indicator of oxidative stress) elevated in cortex and cerebellum in *Gulo*($-/-$)-low mice and in cortex of *Gulo*($-/-$)-sufficient mice. Their study suggested that low vitamin C levels and enhanced oxidative stress are insufficient to impair memory but might be responsible for the motor deficits.

Gulo($-/-$) phenotype: diminished motor function, yet exaggerated response to dopaminergic agonist

By titrating the amount of daily oral vitamin C in the KO mouse, we have generated a mouse model that exhibits dramatic decreases in brain ascorbate (while maintaining normal post-weanling GSH levels); yet, clinically the KO mice appear healthy and unaffected throughout 100 days of postpartum development during which time behavioral phenotyping was completed. Ascorbic acid deficiency caused *Gulo*($-/-$) mice to be slightly less active in spontaneous ambulation and to swim more slowly under some conditions (but not others), adding up to a mild motor and/or reactivity deficit that was more evident in males than females. We saw no evidence of cognitive, anxiety, or sensorimotor-gating problems in the KO mouse, but interestingly they displayed a paradoxical exaggerated response to a dopaminergic agonist—despite being less active when not stimulated.

Exaggerated hyperactivity of KO mouse in response to methamphetamine

This behavioral trait is in striking contrast to their lower-than-control levels of activity on preceding tests and their slower swimming during several phases of water-maze testing. This phenotype might reflect the fact that ascorbate serves as a cofactor for dopamine - hydroxylase in converting dopamine to norepinephrine (Diliberto, Jr. and Allen 1981). Our Fig. 7 data indirectly imply that KO mice have an altered striatal release of dopamine, or a hypersensitive dopamine receptor change, which, under the influence of an indirect sympathomimetic drug that increases dopamine release and inhibits its reuptake, results in up-regulation of dopaminergic activity.

Neostriatal dopamine is known not only to influence locomotor activation, however; dopamine in this region also influences swimming activation (Tamasy *et al.* 1981; van den Bos and Cools 2003; Stuchlik *et al.* 2007; Luthra *et al.* 2009). Therefore, dopamine in the CNS could be a factor in affecting the phenotype of the KO mouse—having a chronic decrease in dopaminergic tone—because dopamine is highly reactive and requires normal antioxidant function to prevent dopaminergic metabolite-induced neuronal damage. If dopamine tone is diminished in *Gulo*($-/-$) mice, this would be expected to induce a compensatory up-regulation of dopamine receptors; this, in turn, would provide increased receptor numbers to respond to a surge in dopamine release caused by the acute methamphetamine exposure.

In the CNS of ascorbic acid-injected awake unrestrained rats, ascorbate in addition to its antioxidant effects was found to modulate phasic changes in striatal excitability induced by glutamate (Kiyatkin and Rebec 1998). Because extracellular levels of ascorbate fluctuate relative to behavioral activation, this neuromodulatory action of ascorbate may contribute to behaviorally relevant changes in locomotor responsivity. Studying the electrical stimulation of cerebral cortex in anesthetized rats given varying concentrations of ascorbate by reverse dialysis, this same lab found that ascorbate increased the striatal glutamate in a concentration-dependent fashion (Rebec *et al.* 2005). It thus appears that the level of extracellular ascorbate might play a role in regulating glutamate transmission and, because glutamate- and dopamine-signaling interact, any alteration in one or both of these could contribute to the behavioral changes seen in *Gulo*($-/-$) mice.

Dopamine and neostriatal function

Our data are consistent with dopaminergic reduction-induced receptor hypersensitivity, leading to hyper-reactivity to dopaminergic stimulants such as methamphetamine. This is consistent with the evidence of decreased initial locomotor activity and slower swimming speed on some of the water maze phases in *Gulo*($-/-$) mice, because these functions are controlled to a significant degree by basal-ganglia-motor nuclei, especially the neostriatum. Locomotion and swimming are impaired by subnormal dopamine function (*vide supra*), which can, in turn, up-regulate dopamine receptors in models of persistent dopaminergic depletion. Neostriatal dopamine and glutamate interact through GABAergic neurons to regulate striatal output; these interactions have been demonstrated to be critical in the mechanism by which methamphetamine induces locomotor activation (Stephans and Yamamoto 1994).

Methamphetamine induces dopamine release and blocks its reuptake—leading to dopamine overflow into the synaptic cleft. Excess extracellular dopamine release stimulates postsynaptic D₁ receptors, thereby increasing GABA release in the striatonigral pathway. Enhanced striatonigral GABA release activates GABA-A receptors in the substantia nigra, which has an inhibitory effect on GABA release in the nigrothalamic pathway. Inhibiting the nigrothalamic pathway disinhibits corticostriatal pathways, thereby increasing glutamate

release, which further augments dopamine release (Mark *et al.* 2004; Mark *et al.* 2007). Because ascorbate is implicated in glutamate regulation (*vide supra*) and glutamate and dopamine interact to control locomotor behavior, the present data are consistent with a role for ascorbate in mediating neostriatal regulation of dopamine-GABA-glutamate signaling. To the extent that ascorbate deficiency perturbs the balance in these pathways, chronic low-ascorbate levels have the potential to disrupt both basal and dopaminergically-stimulated locomotor behavior in the manner seen herein in the *Gulo*($-/-$) mouse.

Vitamin C-deficiency-associated neostriatal dysfunction

Overall, our results are at least partially consistent with ascorbate-deficiency-associated neostriatal dysfunction. This phenotype is similar to, but milder than, the striatal dysfunctions often seen after direct injury to this region—induced by neurotoxic drugs or lesions in which dopamine depletion results in dopamine receptor up-regulation and exaggerated hyperactivity in response to dopaminergic agonists. Limitations of the present experiments include that dopamine and glutamate release and tissue concentrations of these neurotransmitters were not measured, nor were receptor numbers or binding affinity assayed.

Future experiments will be required to test further our hypothesis that ascorbate deficiency acts on neostriatal pathways via a dopamine-glutamate-dependent process. In addition, a more detailed analysis of the nature of the motor and/or emotional dysfunction is warranted. Is the dysfunction exclusively the product of a centrally-mediated signaling abnormality, or is there a peripheral contribution from muscle weakness or defects at the neuromuscular junction? Also, it will be important to distinguish between decreased motor output that is functionally normal vs diminished motor output that is the product of abnormalities—caused by coordination problems, difficulties with movement initiation (as seen in Parkinson disease), problems with ballistic vs fine motor control, or differences in frustration reactivity. These and other factors can only be disentangled by more detailed analyses of each type of movement function, so that we might understand more completely the exact nature of the motor disorder observed in *Gulo*($-/-$) mice.

Conclusions

In summary, the two major antioxidants in mammals are ascorbic acid and GSH. We have titrated the amount of oral ascorbate in *Gulo*($-/-$) mice such that vitamin C levels but not GSH levels were severely diminished in brain; no scurvy or health problems occurred during our behavioral-phenotyping studies that were completed on PND100. We have demonstrated that this degree of ascorbate deficiency in the postnatal brain (in the presence of normal GSH levels) leads to diminished motor functions, yet an exaggerated response to a dopaminergic agonist. This phenotype is consistent with a dopaminergic reduction-induced receptor hypersensitivity, leading to hyper-reactivity to dopaminergic activation. These data suggest that ascorbate deficiency acts on neostriatal pathways via a dopamine-glutamate interaction process. There is a growing clinical appreciation that young children who lack sufficient antioxidants in their diet, including times of malnutrition and infection, might result in CNS defects when these children become adults. Interestingly, our findings in the present study with the ascorbate-deficient *Gulo*($-/-$) mice are consistent with this potential clinical problem.

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Abbreviations

ANOVA	Analysis of variance
ANCOVA	Analysis of covariance
ASR	Acoustic startle response
CNS	Central nervous system
EZM	Elevated zero maze
GABA	-aminobutyric acid
GD	Gestational day
GSH	Reduced glutathione
GULO	<i>L</i> -gulono- γ -lactone oxidase, rate-limiting enzyme in ascorbate acid synthesis
<i>Gulo</i>	Mouse gene encoding GULO
<i>GULO</i>	Human gene encoding GULO
KO	Knockout <i>Gulo</i> ($-/-$) mouse
MWM	Morris water maze
PND	Postnatal day
PPI	Prepulse inhibition
ROS	Reactive oxygen species
SS	Startle signal
WT	Wild-type <i>Gulo</i> ($+/+$) mouse

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Figure 1.

Ascorbate and GSH levels in the brain. Whole brain homogenates prepared from males (N=4) at PND21 and PND60 were assayed for ascorbate (**A**) and GSH (**B**) levels. The *bars* represent means and *brackets* represent S.E.M. * $P < 0.05$ vs WT, # $P < 0.05$ vs WT at PND21.

Figure 2.

Locomotor activity: Mean numbers of photobeam interruptions recorded per 5 min interval during exploration and habituation to a novel environment. Inset: Number of photobeam interruptions averaged across all intervals during the 60-min test session to illustrate the significant main effect of genotype. * $P < 0.05$ vs. WT. Data in all figures represent least-squares means $\pm$ least-squares S.E.M. Numbers of (males/females): WT = 42 (23/19); KO = 45 (18/27).

Figure 3. MWM (cued). *Left:* Latency (in seconds) to reach the platform on Day-1 (means $\pm$ S.E.M. of six trials) with both the start and goal in fixed positions. *Right:* Latency to reach the platform on Days 2–6 (mean $\pm$ S.E.M. of two trials/day. * P <0.05, ** P <0.01 vs WT. The numbers of (males/females) are same as in Fig. 2.

Figure 4.

MWM (hidden). Mean path length (cm) to reach the platform during acquisition (*left*), reversal (*middle*), and shift (*right*) panels. Data represent the means of four trials/day during each phase of testing. Learning curves are evident in both groups across all the days but no significant effects of genotype were seen. Numbers of (males/females): WT = 39–42 (23/16–19); KO = 40–45 (16–18/24–27).

Figure 5.

MWM swim speed (in cm per second) during training. Average swimming speed (means $\pm$ S.E.M.) during hidden platform trials on the fixed-platform version of the test. **A**, swim speed during the acquisition phase; **B**, swim speed during the reversal phase. **C**, swim speed during the shift phase. $^{\dagger}P < 0.06$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs WT. The numbers of (males/females) are same as in Fig. 5.

Figure 6.

MWM swim speed (in cm per second) on probe trials. Least-squares mean ($\pm$ S.E.M.) swim speeds during probe trials for three phases of MWM testing: **A**, Acquisition, **B**, Reversal, **C**, Shift. ** $P < 0.01$ vs WT. The numbers of (males/females) are same as in Fig. 5. **M**, male. **F**, female.

Figure 7.

Locomotor activity before and after drug challenge. Mean ($\pm$ S.E.M.) numbers of photobeam interruptions per 5 min interval for 30 min prior to methamphetamine administration (1.0 mg/kg) and for 120 min post-methamphetamine administration. * $P < 0.05$, ** $P < 0.01$ vs WT. Numbers of (males/females): WT = 39 (23/16); KO = 41 (18/23).