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Effect of chronic glutathione deficiency on the behavioral phenotype of *Gclm*($-/-$) knockout mice

Ying Chen^{1,*†}, Christine P. Curran^{1,*§}, Daniel W. Nebert¹, Krishna V. Patel¹, Michael T. Williams², and Charles V. Vorhees^{2,‡}

¹Department of Environmental Health, and Center for Environmental Genetics (CEG) University of Cincinnati Medical Center, Cincinnati, Ohio 45267-0056

²Department of Pediatrics, Division of Neurology, University of Cincinnati Medical Center, and Cincinnati Children's Research Foundation, Cincinnati, OH 45229

Abstract

Enhanced oxidative stress or deficient oxidative stress response in the brain is associated with neurodegenerative disorders and behavioral abnormalities. Previously we generated a knockout mouse line lacking the gene encoding γ -glutamylcysteine ligase modifier subunit (**GCLM**). *Gclm*($-/-$) knockout (**KO**) mice are viable and fertile, yet exhibit only 9–35% of wild-type levels of reduced glutathione (**GSH**) in tissues, making them a useful model for chronic GSH depletion. Having the global absence of this gene, KO mice—from the time of conception and throughout postnatal life—experience chronic oxidative stress in all tissues, including brain. Between postnatal day (**P**) 60 and P100, we carried out behavioral phenotyping tests in adults, comparing male and female *Gclm*($-/-$) with *Gclm*($+/+$) wild-type (**WT**) littermates. Compared with WT, KO mice exhibited: subnormal anxiety in the elevated zero maze; normal overall exploratory open-field activity, but slightly more activity in the peripheral zones; normal acoustic startle and prepulse inhibition reactions; normal novel object recognition with increased time attending to the stimulus objects; slightly reduced latencies to reach a random marked platform in the Morris water maze; normal spatial learning and memory in multiple phases of the Morris water maze; and significantly greater hyperactivity in response to methamphetamine in the open field. These findings are in general agreement with two prior studies on these mice and suggest that the brain is remarkably resilient to lowered GSH levels, implying significant reserve capacity to regulate reactivity oxygen species—but with regional differences such that anxiety and stimulated locomotor control brain regions might be more vulnerable.

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[‡]Correspondence: Charles V. Vorhees, Ph.D., Professor, Division of Neurology (MLC 7044), Cincinnati Children's Research Foundation, Department of Pediatrics, University of Cincinnati College of Medicine, 3333 Burnet Avenue, Cincinnati, OH 45229-3039 USA. Tele 513-636-8622; Fax 513-636-3912. charles.vorhees@cchmc.org.

^{*}These two authors contributed equally to this project.

[†]Current address: Department of Pharmaceutical Sciences, University of Colorado AMC, Aurora, CO 80045

[§]Current address: Department of Biological Sciences, Northern Kentucky University, Highland Heights, KY 41099

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None.

COI statement

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Keywords

acoustic startle response; elevated zero maze; GSH; reduced glutathione; locomotor activity; Morris water maze; novel object recognition

Introduction

Oxidative stress occurs when oxygen free radicals production exceeds cellular defense mechanisms (Dalton et al., 1999). The brain generates large amounts of reactive oxygen species (**ROS**) during cellular metabolism but has antioxidant systems to maintain redox homeostasis. The primary antioxidants are glutathione (**GSH**) (Dalton et al., 2004), ascorbate (**vitamin C**) (Harrison and May, 2009), lipoic acid (De Araujo et al., 2011), uric acid (Amaro et al., 2008), carotenoids (Obulesu et al., 2011), tocopherols/tocotrienols (**vitamin E**) (Clarke et al., 2008), ubiquinone/ubiquinol (**coenzyme Q**) (Dhanasekaran and Ren, 2005), and melatonin (Reiter et al., 2010).

GSH is the most abundant cellular non-protein thiol (2–4 mM in neurons and slightly higher in glia) (Rice and Russo-Menna, 1998). GSH scavenges free radicals and serves as a cofactor for antioxidant enzymes such as glutathione peroxidases and glutathione *S*-transferases (Meister, 1991). GSH is also important in defense against toxicity from endogenous and exogenous electrophiles (Monks et al., 1999; Fonnum and Lock, 2004) and serves as a signaling molecule through its redox regulation of protein functions (Dalle-Donne et al., 2007). GSH may modulate synaptic transmission via redox-sensitive proteins—including NMDA receptors (Sucher and Lipton, 1991), GABA-A receptors (Amato et al., 1999), calcium-activated K⁺ channels (Tang et al., 2003), voltage-gated calcium channels (Brown et al., 2005), and glutamate transporters (Volterra et al., 1994; Trotti et al., 1997).

GSH is synthesized by γ -glutamate combining with cysteine, catalyzed by γ -glutamylcysteine ligase (**GCL**), followed by addition of glycine via glutathione synthase. The GCL holoenzyme is a heterodimer of the 72.8-kDa catalytic subunit (**GCLC**) and the 30.8-kDa modifier subunit (**GCLM**). GCLM maintains GSH homeostasis by optimizing the catalytic efficiency of the holoenzyme properties GCLC (Chen et al., 2005). Both genes are expressed in mouse embryo, fetus, yolk sac, and placenta (Diaz et al., 2002; Diaz et al., 2004). In liver, lung, pancreas, erythrocytes, and plasma, GSH levels in *Gclm*($-/-$) knockout (KO) mice are 9–16% of WT (Yang et al., 2002). Except when challenged with oxidant stress, *Gclm*($-/-$) animals are viable and fertile and exhibit no overt phenotype, making them a useful model of GSH deficiency (McConnachie et al., 2007). Fetal fibroblasts derived from *Gclm*($-/-$) mice are sensitive to oxidative stress and oxidant-induced cell death (Yang et al., 2002). Primary astrocytes from *Gclm*($-/-$) mice show diminished cell viability and higher rates of apoptosis when exposed to an oxidizing agent, the polybrominated flame retardant DE-71 (Giordano et al., 2008).

Disorders associated with GSH deficiency include Parkinsonism, amyotrophic lateral sclerosis, Alzheimer disease, epilepsy and other neurodegenerative diseases (Aoyama et al., 2008). Individuals carrying rare polymorphic variants of the *GCLC* gene, with low enzymatic activity and decreased intracellular GSH, manifest symptoms ranging from hemolytic anemia to neurological disorders (Ristoff and Larsson, 1998). Other polymorphisms of the *GCLC* and *GCLM* genes, that result in modestly lowered GSH levels, have been associated with schizophrenia (Tosic et al., 2006).

The major objective of this study was to assess the causal relationship between chronic GSH deficiency and behavioral abnormalities using male and female adult *Gclm*($-/-$) mice. We previously phenotyped other genetically-modified mice (Schaefer et al., 2009; Curran et al.,

2011; Skelton et al., 2011) using a battery of behavioral tests that included a multi-phase version of the Morris water maze known to detect subtle differences in hippocampal dependent spatial learning and memory (Vorhees and Williams, 2006). Herein we determined the effect of chronic GSH deficiency using a similar approach.

Materials and Methods

Animals

Gclm($-/-$) knockout mice were previously generated in our lab (Yang et al., 2002). These mice were backcrossed eight times to C57BL/6J (**B6**) mice to establish a >99.8% B6 background; *Gclm*($+/-$) heterozygotes were bred in order to generate WT controls and KO offspring. Plug-positive females were removed from breeding and housed separately throughout gestation and lactation until dams were removed from their litters on postnatal (P) 28 (birth was counted as P0). Offspring were genotyped and WT and KO mice within each litter were retained for testing. Behavioral testing was conducted between P60–P100. Mice were maintained on regular lab chow and filtered water ad lib on a 14:10 light:dark cycle (lights on at 700 h). The protocol under which this project was conducted was approved by the Institutional Animal Care and Use Committee.

GSH, GSSG, and ascorbate assays

Whole brain was collected from WT and KO mice (N=4 per group) at age P60. Mice were euthanized by CO₂, whole brain was excised, snap-frozen in liquid nitrogen, and stored in -80° C. At the time of assay, frozen whole brain was ground in nine volumes of ice-cold redox-quenching buffer (20 mM HCl and 5 mM diethylenetriaminepentaacetic acid) containing 5% trichloroacetic acid. This mixture was then centrifuged at $12,000 \times g$ for 10 min, and the resulting supernatant was used for biochemical measurements. GSH and GSSG levels were determined spectrophotofluorometrically, using the fluorescent probe α -phthalaldehyde, as described (Senft et al. 2000). Another aliquot of the supernatant was treated with dithionite to convert GSSG to GSH, and the total GSH + GSSG was determined. Ascorbate levels were determined by spectrophotometric measurement of ferritin iron released by ascorbate as described (Zannoni et al. 1974). Briefly, an aliquot of the supernatant was mixed sequentially with 85% H₃PO₄, 1% 2,20-bipyridine, and 3% ferric ammonium sulfate; after incubation at room temperature for 30 min, absorbance was determined at 525 nm. Concentrations of GSH, GSSG, and ascorbate in each sample were interpolated from known standards. Results were reported as μ mole/g tissue.

Behavior

Gclm($-/-$) mice were compared with WT mice using a battery of behavioral tests (Vorhees, 1996; McIlwain et al., 2001; Bailey et al., 2006; Paylor et al., 2006; Crawley, 2007; Crawley, 2008). Not more than one male and one female/litter/genotype were tested (16–20/group) beginning on P60 as follows: Week-1 = elevated zero maze (**EZM**), open-field locomotor activity, acoustic startle response (**ASR**) with prepulse inhibition (**PPI**). Week-2 = novel-object-recognition (**NOR**). Week-3 = Morris water maze (**MWM**) cued; Week-4 = MWM hidden acquisition. Week-5 = MWM hidden reversal. Week-6 = MWM hidden shift. Week-7 = locomotor activity with (+)-methamphetamine (1 mg/kg; subcutaneously) challenge. All tests were performed during the light portion of the light:dark cycle. Not all litters were perfectly balanced by sex and genotype making a balance-by-litter experimental design impossible; however, by testing no more than one male and one female of any one genotype from any given litter, control of any litter effects was maintained.

Monoamine neurotransmitter

Dopamine, serotonin and their respective metabolites DOPAC and 5-HIAA were measured in prefrontal cortex, hippocampus, and neostriatum (caudate and putamen) using the methods described (Grace et al., 2010). Brains were collected from *Gclm* WT and KO mice (N = 6 to 14 per group). In brief, tissue concentrations of DA, DOPAC, 5-HT, and 5-HIAA were quantified using high-pressure liquid chromatography with electrochemical detection. Tissue weights were determined prior to homogenization in 50 volumes of 0.2 N perchloric acid and centrifuged for 6 min at $10,000 \times \text{RCF}$. Aliquots of 20 μl were injected onto a C₁₈-column (MD-150, 3 $\times$ 150 mm; ESA, Chelmsford, MA) connected to a Coulochem detector (25A, Chelmsford, MA) and an integrator recorded the peak heights for each injection. The potentials of the E1 and E2 on the analytical cell (model 5014B) of the Coulochem were -150 and $+160$ mV, respectively. The mobile phase consisted of 35 mM citric acid, 54 mM sodium acetate, 50 mg/l of disodium ethylenediamine tetraacetate, 70 mg/l of octanesulfonic acid sodium salt, 6% (v/v) methanol, 6% (v/v) acetonitrile, pH 4.0, and pumped at a flow rate of 0.4 ml/min. Quantification of analytes was calculated on the basis of standards.

Elevated zero-maze

The EZM is a circular runway (105-cm diameter), 72 cm above the floor with a 10-cm circular path divided equally in four quadrants, designed to assess anxiety based on avoidance of open spaces. Two opposite quadrants have 28-cm walls and two have 1.3-cm clear acrylic curbs. Mice were video-recorded for 5 min and later scored by an observer blind to treatment group for the following behaviors: Latency to leave the closed quadrant, time-in-open, head-dips, and zone-crossings (Shepherd et al., 1994).

Locomotor activity

Open-field arenas were 40 $\times$ 40 cm with 16 infrared LED-photocells in x- and y-planes spaced 2.5 cm apart. Mice were tested for 1 h (Accuscan Instruments; Columbus, OH) and photobeam interruptions were analyzed in 5-min intervals. The test assesses exploration and habituation, as well as fear of open spaces—as reflected by central zone activity.

Acoustic startle response with prepulse inhibition

SR-LAB test chambers (San Diego Instruments; San Diego, CA) were used with a 5-min acclimation period with background mixed frequency noise of 68 dB. Following acclimation, mice received a 4 $\times$ 4 Latin square protocol of four trial types repeated three times: no-stimulus, startle signal (SS), 74-dB prepulse (PP) + SS, or 76-dB PP + SS. The inter-trial interval was 8 s and the inter-stimulus interval was 70 ms (from PP onset to SS onset). The startle signal was a mixed-frequency white noise burst (120 dB SPL, 20 ms). Prepulse signals were also 20 ms duration. Peak response amplitude (V_{max}) was the dependent measure analyzed. This test assesses the basic acoustic startle reflex pathway as modified by higher brain regions using PPI, which reflects sensorimotor gating and, as such, one aspect of attention.

Novel object recognition

Mice were habituated to arenas (91-cm diameter) for 4 days. On the test day, two different identical objects were presented until 30 s of observation had accrued. Observation by the animal was defined as being within 1 cm of the object and oriented toward it, but excluded climbing on it. This method was used in order to try to equalize attention time to objects, so that the denominator in all group comparisons is the same (Clark et al., 2000). One hour later, an exact copy of the familiar object and a novel object were presented until 30 s of observation had accrued (up to 10 min). Objects were counter-balanced to avoid confounding by object preference. The dependent variable was the percent time attending to

the novel versus the familiar object. This is a test of memory, based on retention and recognition of a previously seen object.

Morris water maze

The tank was 122-cm diameter and the method similar to that described (Vorhees and Williams, 2006). Day-1: six cued trials with the start and platform positions fixed. The purpose of this procedure was to teach the mice to find, climb on, and remain on the platform until removed. Days 2–6: two trials/day each with a random start and random platform position. During all cued trials, curtains were closed around the pool to obscure distal cues. The escape platform was 10-cm in diameter and contained an orange ball mounted 10 cm above the surface on a metal rod to provide a direct proximal cue to the platform's location. Following completion of cued trials, mice received three phases of hidden-platform testing. Each of these phases consisted of four trials/day for 6 days with a 30-s probe trial on Day-7 (see (Curran et al., 2011)). Each phase used a progressively smaller platform (10, 7, 5 cm in diameter for acquisition, reversal, and shift, respectively). During acquisition the platform was located in the southwest quadrant, during reversal in the northeast quadrant, and during shift in the northwest quadrant—relative to the position where the experimenter stood (arbitrarily designated as south). The platform was positioned halfway between the center and wall of the tank. On hidden platform trials, start positions were pseudo-randomized with the restriction that on each day there was one trial from each of four possible distal start locations. To avoid start positions close to the target quadrant, only distal start positions were used, *i.e.*, two cardinal and two ordinal positions farthest from the platform, *e.g.*, when the platform was in the southwest position, start locations were either cardinal (north or east) or ordinal (northwest or southeast). Data were collected automatically by an overhead video camera attached to a closed-circuit monitor and a computer with tracking software (Smart-track, San Diego Instruments; San Diego, CA). Extracted data files were analyzed on platform trials for latency to reach the platform, cumulative distance to the platform, path length, and swim speed and on probe trials with the platform removed for platform-site crossovers, average distance to the platform site, quadrant preference, and swim speed. This is a test of spatial or allocentric learning with probe trials, which reflects spatial reference memory.

Locomotor activity with drug challenge

Prior to drug challenge, mice were re-habituated to the open-field test chambers with no drug given for 30 min. After 30 min, the test was briefly interrupted and each mouse removed, injected subcutaneously with (+)-methamphetamine HCl (1.0 mg/kg, prepared as the free base in a volume of 1 ml/kg of saline), and returned to the test chamber and activity monitored for an additional 120 min.

Statistical analyse

Behavioral data were analyzed using mixed-linear analysis of variance (**ANOVA**) with repeated-measures, or by analysis of co-variance (**ANCOVA**) in the case of locomotor activity after drug challenge using prechallenge activity as the covariate. Data were analyzed using SAS procedures (version 9.2, SAS Institute; Cary, NC). Follow-up analyses of interactions were conducted using slice-effect ANOVAs. Because there were only two groups, *a posteriori* pairwise group comparisons were analyzed by t-test for independent samples. Data on antioxidant levels were analyzed by t-test for independent samples (two-tailed). Neurotransmitter data were analyzed by general linear model ANOVA. Data are presented as least square (LS) means $\pm$ SEM. Results were considered significant if $P \leq 0.05$.

Results

GSH and GSSG levels in brain

At the age (P60) when mice were evaluated by behavioral tests, GSH and GSSG levels (Fig. 1A & B) in whole brain from *Gclm*($-/-$) KO mice were 35% and 58%, respectively, of that from WT mice. The resulting GSH/GSSG ratio was diminished ~40% in KO mice compared with WT mice (Fig. 1C); these data confirm a state of chronic oxidative stress in *Gclm*($-/-$) brain. We did not observe any compensatory increase of ascorbate, another major antioxidant that interplays with GSH (Mårtensson and Meister, 1991), in the KO brains (Fig. 1D).

Body weight

Gclm($+/+$) and *Gclm*($-/-$) offspring as adults showed slight but significant bodyweight differences ($F(1,72) = 6.40$, $P < 0.02$). At P100, the LS mean $\pm$ SEM of male WT and KO mice were 26.3 ± 0.4 g ($N=16$) and 25.6 ± 0.4 g ($N=18$), respectively, and female WT and KO mice were 20.7 ± 0.4 g ($N=18$) and 19.3 ± 0.4 g ($N=21$), respectively. KO mice showed no overtly different phenotype and appeared as healthy as WT mice throughout the experiment.

Monoamine neurotransmitters

There were no significant differences in levels of dopamine, serotonin or their metabolites in the three brain regions analyzed (neostriatum, hippocampus, and prefrontal cortex) (data not shown).

EZM

There was a significant genotype main effect for time-in-open ($F(1,63) = 4.88$, $P < 0.05$) but no interaction with sex (Fig. 2A). Similarly, there was a main effect of genotype for head-dips ($F(1,63) = 6.16$, $P < 0.02$) (Fig. 2B). There was also a significant main effect of genotype for latency to first open-zone entry ($F(1,63) = 3.88$, $P < 0.05$). For latency to first open-zone entry, there was also a significant genotype $\times$ sex interaction ($F(1,63) = 6.37$, $P < 0.02$). Slice-effect ANOVA on the interaction showed no differences between males, but a significant difference between females ($P < 0.01$; Fig. 2C) in which female KO mice had shorter latencies to first open-zone entry than female WT mice. For the number of zone crossings, there was no significant genotype or genotype $\times$ sex interaction (Fig. 2D).

Open-field locomotor activity

For the 1-h open-field locomotor activity test, there were no significant effects of genotype, but a trend was seen ($P < 0.09$) in which KO mice were slightly more active than WT mice. Sex, interval, and the sex $\times$ interval effects were significant but did not interact with genotype. We also analyzed central and peripheral distance (successive beam interruptions converted to cm) using a genotype $\times$ region $\times$ sex interval ANOVA. There were main effects of gene ($F(1,77) = 5.24$, $P < 0.03$), sex ($P < 0.01$), region ($P < 0.0001$), and interval ($P < 0.0001$). There were also significant interactions of genotype $\times$ region ($F(1,77) = 6.11$, $P < 0.02$), region $\times$ sex ($F(1,77) = 12.07$, $P < 0.001$), sex $\times$ interval ($F(11,847) = 3.87$, $P < 0.001$), region $\times$ interval ($F(11,847) = 112.95$, $P < 0.0001$), region $\times$ interval $\times$ sex ($F(11,847) = 4.36$, $P < 0.0001$); the remaining 2-, 3-, and 4-way interactions were not significant. In order to follow-up the genotype $\times$ region interaction, separate ANOVAs were conducted for each region.

No significant effects of genotype or genotype interactions with sex or interval on central distance were found (Fig. 3A). With regard to peripheral distance, there was a significant

genotype main effect ($F(1,77.1) = 5.87, P < 0.02$) and genotype $\times$ interval interaction ($F(11,697) = 1.83, P < 0.05$). Slice-effect ANOVAs for each interval showed differences at multiple intervals and overall (Fig. 3B). KO animals were more active in the periphery than WT controls.

ASR with PPI

There were no significant effects of genotype or interaction of genotype with other factors (sex or prepulse) on acoustic startle response amplitude, with or without a prepulse present (not shown), but there was a significant inhibitory effect of prepulse that was intensity-dependent ($F(2,152) = 259.34, P < 0.0001$).

NOR

During the familiarization trial, KO and WT mice showed no significant difference in their preference for one object over the other, *i.e.*, both groups on average spent approximately 50% of their time attending to each of the two identical objects. On the novelty test trial, no significant effect of genotype on novel object preference and no genotype $\times$ sex interaction (Fig. 4A) were found, *i.e.*, of the 30 s spent attending to the objects, both groups equally preferred the novel (65%) compared with the familiar (35%) object. However, it was noted that an unusual number of animals failed to complete the task, *i.e.*, failed to accumulate 30 s of exploration of the objects during the test period. We therefore analyzed the proportion of animals failing to complete the test (Fisher's Exact Test). There was a significant difference (Fig. 4B) in failed trials as a function of genotype ($P < 0.03$): 54.0% of WT mice failed to accumulate 30 s of object time, whereas only 27.9% of KO mice failed to do so.

MWM, cued platform

Day-1 involved six trials, with the same start and goal positions. The purpose was to train mice to remain on the platform because mice on early trials often jump off the platform and resume swimming the first few times they find it. No significant genotype effect or genotype $\times$ trial or sex interaction was seen (Fig. 5A).

Days 2–6 involved two trials per day, with the start and goal randomly positioned for each trial. The purpose was to train the mice to search for the platform using only the cue because no other strategy provided reliable information as to the platform's location. There was a significant genotype effect ($F(1,75.1) = 3.81, P = 0.05$; Fig. 5B) in which KO mice, regardless of sex, took less time to reach the platform than WT mice. There were no significant genotype interactions with sex, trial, or the combination of both. There was a sex main effect ($P < 0.02$) because males had shorter latencies than females of both genotypes.

MWM, hidden platform

There were no genotype 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 (Figs. 6A & C for cumulative distance), *i.e.*, all groups showed typical mouse learning curves. There was no genotype main effect on reversal (phase-2) for path length or latency, but there was a significant genotype $\times$ day effect on cumulative distance from the platform [$F(5,268) = 2.22, P < 0.05$]. Genotype $\times$ sex slice-effect ANOVAs on each day failed to show a significant genotype or genotype $\times$ sex interaction on any single day (Fig. 6B). For swim speed, there were no significant main effects or interactions on any phase of the test suggesting that KO mice had no sensorimotor or motivation deficits that might interfere with their ability to locate the platform.

MWM, probe trials

We measured average distance to the platform site, number of site-crossovers, swim speed, and percent time and percent distance in the target quadrant. For average distance, there were no significant effects of genotype or interactions on the acquisition, reversal, or shift probe trials. Similarly, there were no genotype effects on probe swim speed, or percent time or percent distance in the target quadrant.

For crossovers (number of times a mouse swam over the spot where the platform had previously been), there were no genotype effects found on the acquisition or shift probe trials, but an effect was observed on the reversal probe trial. Whereas the genotype main effect was not significant, the genotype $\times$ sex interaction was ($F(1,72) = 4.92$, $P < 0.03$; Fig. 7). Slice-effect ANOVA on males showed no significant genotype effect, whereas for females the genotype effect was significant. KO females had more crossovers than WT females ($P < 0.05$), suggesting that they were better than WT females at remembering the location where the platform had been previously.

Locomotor activity with drug challenge

During the 30-min re-habitation phase, we found no significant main effect of genotype or genotype $\times$ interval, genotype $\times$ sex, or genotype $\times$ sex $\times$ interval.

For the post-challenge activity, there was a significant main effect of genotype ($F(1,74.5) = 7.47$, $P < 0.01$; Fig. 8) but no genotype interactions with sex, interval, or the combination of all three factors. KO mice showed an exaggerated hyperactivity in response to methamphetamine compared with WT mice. The effect was maximal from 20 to 70 min post-challenge but was statistically significant only when averaged across all intervals (Fig. 8, inset).

Discussion

In the present study we used a test battery to obtain a broad spectrum of possible behavioral effects in a mouse model with chronic oxidative stress from GSH reduction. Because GCLM modifies the regulation of GCLC and therefore regulates GSH levels that serve to buffer ROS, we postulated that *Gclm*($-/-$) KO mice would be at greater risk than WT mice for enhanced CNS damage—given the dramatic decreases in brain GSH (Fig. 1). In contrast to this prediction, KO mice performed within the range of WT mice on most of the tests given and, on a few tests, performed better than WT mice. For example, KO mice displayed better completion rates in the NOR test, had shorter escape latencies on random cued trials in the MWM, and (female) KO mice had more site crossovers on the reversal-probe trial. Compared with WT mice, there were some tests that suggested abnormalities in KO mice: mild hyperactivity in the peripheral zones of the open-field test, diminished anxiety in the EZM, and exaggerated hyperactivity in response to a challenge dose of methamphetamine. These findings represent a moderate behavioral phenotype, which was unexpected—given the degree of GSH depletion in the brain of KO mice.

Our results agree, in some respects, with findings from another study using the *Gclm*($-/-$) mouse (Steullet et al., 2010). This group reported increased oxidative stress, reduced GABAergic signaling and a significant decrease in parvalbumin-immunoreactive interneurons in the CA3 and dentate gyrus of the ventral hippocampus with no damage seen in the dorsal hippocampus. This pathology is consistent with our behavioral phenotype, because it would not affect spatial learning and memory, but could produce behaviors associated with the onset of schizophrenia which has been linked to reduced GSH in humans (Tosic et al. 2006).

Steullet et al. (2010) used a battery of behavioral tests as well. They tested object recognition as we did, except they evaluated both novel-place and novel-object recognition and they used a 10-min retention test interval compared with our 1-h retention test. They found that KO mice explored objects more than WT mice even during the familiarization phases; this finding is somewhat consistent with our finding that more KO than WT mice reached the object-exploration criterion. Similar to our experiment, they found no evidence of impaired novel object preference during retention, suggesting no impairment of object memory.

During appetitive alternation T-maze testing, used as a test of spatial working memory, Steullet et al. (2010) reported no significant differences between KO and WT mice. We did not test for working memory; therefore no comparison of this outcome is possible. They also used the MWM, as we did, to test for spatial learning and they included a reversal-learning phase as we did. Similar to our data, they found no evidence of deficits in MWM cued- or hidden-platform spatial learning on acquisition or reversal phases; Steullet et al. (2010) also showed no evidence of impaired reference memory on the post-acquisition probe trial. Hence, these data are consistent with ours, in that KO mice exhibit no impairment of cued- or hidden-platform performance, including no effects on probe trial tests of memory.

Steullet and coworkers (2010) used several measures of anxiety. One was a novel food suppression test. They found a trend in KO mice to approach food in a novel environment more quickly than WT mice. In another test, they used the elevated plus maze (EPM) and found, as we did in the EZM, that KO mice entered open areas more than WT mice. To further test anxiety, they used the light-dark test and found that KO mice made more zone transitions and spent more time in the light than did WT mice; this is consistent with the EPM and EZM data—indicating that KO mice are less anxious or have increased risk taking compared with WT mice.

Exhibiting less evidence of anxiety may be a positive or negative characteristic. It may be a positive attribute in the context of a preexisting anxious state; however, when an organism is euthymic, exhibiting abnormally low anxiety may be maladaptive and lead to risk-taking or absence of adaptive fear of high risk environments, which for rodents can be open spaces. For rodents, such behavior might place them at greater risk to predation; in humans, lack of appropriate fear is often associated with excessive risk-taking that can lead to dangerous actions that may result in injury to themselves or others.

Steullet et al. (2010) also tested *Gclm*($-/-$) mice for conditioned, as well as approach-avoidance, fear. Day-1 of the procedure was to expose mice to a neutral stimulus (tone) paired with foot shock as conditioning. On day-2 the mice were placed back in the same chamber and assessed for contextual fear. On day-3, mice were placed in a different chamber for an adaptation period with no tone, then tone was presented and freezing behavior recorded. They found that KO mice showed decreased freezing to both the conditioned context and cue—suggesting less conditioning, or reduced fear, compared with WT mice. This is consistent with the lowered anxiety found using the tests of anxiety described above.

More recently, another group reported on the behavioral phenotype of *Gclm*($-/-$) KO mice, also using a behavioral test battery (Cole et al., 2011). They compared KO with WT mice on tests of ASR/PPI, rotorod, automated open-field, and MWM acquisition and reversal. Three of these tests are nearly identical to what we did, *viz.*, ASR/PPI, automated open-field, and MWM. Similar to our study, they found no changes in KO mice on ASR/PPI, open-field, or MWM acquisition, reversal or probe, even though several procedural details differed from our methods. They did not separate open-field activity into central and peripheral sub-

regions or use a psychostimulant drug-challenge in the open-field, thus it is unknown if the mice they tested would have similar effects to those observed in this study. They tested their mice on a rotarod for motor coordination (which was not in our battery of tests), and they found no differences.

Cole et al. (2011) did not carry out tests of anxiety or conditioned fear and, hence, did not detect the diminished anxiety or fear-conditioning that we and Steullet et al. (2010) found. On balance, across the two previous behavioral phenotyping studies and the present data, there is a large measure of agreement. All three studies find that *Gclm*($-/-$) KO mice show the following: no evidence of cognitive/learning/memory impairment, no motoric deficits measured as spontaneous locomotion, forced rotarod walking, or changes in swimming ability (indexed as swim speed); however, KO mice were found to display decreased anxiety and fear-associated learning, as well as an altered response to a dopaminergic stimulant.

These experiments—as in the cases of Steullet et al. (2010) and Cole et al. (2011)—were behavioral characterizations and were done without knowledge that these other similar studies were in progress. Ours, by being reported last and without benefit of seeing the findings of the other two, may appear confirmatory, to which we would concur. However, interlaboratory confirmation of a genetic phenotype is important and strengthens the previous observations. In addition, the present data add outcomes not found in the previous data on this mouse model. First, we tested both males and females and showed that for *Gclm*-deletion, there are no striking sexually dimorphic behavioral differences caused by disruption of this gene, and we found that males and females are similarly affected. More significantly, we found that *Gclm* KO mice over-respond to a moderate dose of methamphetamine, a drug that predominantly induces dopamine overflow. Methamphetamine in adult animals works through four mechanisms: (1) inhibiting the dopamine transporter (DAT) which blocks DA reuptake, (2) inhibiting VMAT2 which blocks synaptic vesicle reuptake, (3) reversing DAT resulting in DA efflux, and (4) inhibiting MAO metabolism of DA. The fact that *Gclm*($-/-$) mice have a differential response to a drug acting through these mechanisms provides an avenue for follow up experiments to determine if DA is central to how disruption of the *Gclm* gene affects CNS function.

The findings also offer possible clues to brain antioxidant mechanisms. First, ablation of the *Gclm* gene lowers brain GSH levels (Fig. 1). If this were the brain's sole or principal antioxidant control mechanism—one would expect severe neurotoxicity and neurobehavioral deficits. Because this was not observed in three independent studies, loss of GCLM suggests that the brain has redundant antioxidant systems to protect itself from ROS damage, even when the GSH system is compromised. Although brain ascorbate is not compensatorily increased, neither is it decreased in KO mice (Fig. 1); thus, existing ascorbate levels might provide partial protection against GSH loss from ROS damage in a manner similar to that shown in newborn rats (Mårtensson and Meister, 1991). An alternative possibility for protection against oxidants might be melatonin, which has been shown to protect against oxidative stress particularly in dopaminergic pathways (Ma et al., 2009; Hashimoto et al., 2011). If this is the case, the altered response to methamphetamine suggests that such protection is incomplete.

The data further show that, despite the brain's resilience to lowered GSH levels, not all neural pathways are protected. Instead, the findings suggest that there are regions that are vulnerable to chronic GSH reduction. Given the changes in anxiety and conditioned fear, that both depend on the amygdala, the data across studies suggest that this region is less capable of buffering the effect of ROS exposure than other regions. Interestingly, methamphetamine depletes GSH in the amygdala of mice (Achat-Mendes et al., 2007) and

increases levels of GSH-related proteins in rats (Iwazaki et al., 2008), suggesting an important role for GSH in regulating function in this brain region. The neostriatum, on the other hand, showed a different effect: under basal conditions when the mouse was in a novel environment, no effects in total movement were observed; however, a small and variable increase in movement in the perimeter of the apparatus was seen, but when locomotion was augmented, driven by exposure to the dopaminergic agonist methamphetamine, a much larger difference emerged. This differential response is unlikely to be attributable to nonspecific effects because—even though we did not include a saline-challenged control condition—we previously showed that such a control (saline injection) induces no significant locomotor response (Ehrman et al., 2006).

Human disorders associated with GSH depletion include some that are neurodegenerative, such as amyotrophic lateral sclerosis, Parkinson disease, and Alzheimer disease (Aoyama et al., 2008), pathologies with overt symptoms not found in the *Gclm*-null mice. Therefore, it may be important to test aged *Gclm*($-/-$) animals to determine whether or not greater impairments are found following life-long GSH depletion. It is also possible that humans with mutations that deplete GSH might be at higher risk to exposure from neurotoxic agents that increase ROS. For example, *Gclm*($-/-$) mice are more susceptible to the pollutant 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (Chen et al., 2011), and rats having decreased GSH are more susceptible to manganese neurotoxicity (dos Santos et al., 2010).

Further experiments may profit by using the *Gclm*($-/-$) mouse in combination with other models of decreased antioxidant capability, such as the *Gulo*($-/-$) knockout mouse that cannot synthesize the antioxidant ascorbic acid. We have recently found that *Gulo*($-/-$) mice also exhibit a behavioral phenotype that is relatively subtle, yet different in many respects from that seen herein with the *Gclm*($-/-$) mouse (Chen et al., 2012). However, there is one striking commonality between the *Gclm*($-/-$) and *Gulo*($-/-$) KO mice: both show an exaggerated hyperactivity in response to methamphetamine. This similarity suggests that the neostriatum is particularly susceptible to ROS-induced neuronal injury. Given that the striatum is a region of high dopamine abundance, it may be conjectured that dopaminergic terminals are more susceptible to neurotoxic effects than terminals containing other neurotransmitters. Or, it may be that, because dopamine and glutamate interact to facilitate the release of one other, and high glutamate concentrations can cause excitotoxicity, perhaps lowered GSH places other neurotransmitter cell-types at risk—culminating in dopamine receptor up-regulation and increased response to methamphetamine. Crossing the *Gclm*($-/-$) and *Gulo*($-/-$) mouse lines to create a double-knockout mouse could be one way to test this hypothesis, because ablation of both genes may create a mouse with an extreme locomotor response to methamphetamine and perhaps to other pharmacological probes as well. Given the well-known interactions between GSH and ascorbate (Mårtensson and Meister, 1991), we would also gain additional information concerning these important antioxidant defense systems in the CNS.

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Highlights

- Oxidative stress is associated with brain and behavioral abnormalities
- We generated mice lacking glutamylcysteinyl ligase modifier GCLM which reduces glutathione (GSH) antioxidant activity
- KO mice exhibited anxiety changes in the elevated plus maze and increased peripheral zone locomotor activity
- KO mice had shortened latencies to reach the cued platform in the Morris water maze but were normal on hidden platform trials of spatial learning and memory
- KO mice showed exaggerated hyperactivity in response to methamphetamine challenge
- We conclude that some brain regions are resilient low GSH, while others that influence anxiety and locomotion are more susceptible

Fig. 1. GSH and ascorbate levels in brain. Whole-brain homogenates prepared from males (N=4) at P60 were assayed for GSH, GSSG, and ascorbate levels. The *bars* represent means and *brackets* represent S.E.M. * $P < 0.05$ vs WT.

Fig. 2. Elevated zero maze (5 min). **(A)** Percent time spent in open quadrants; **(B)** number of head-dips over the side of open quadrants; **(C)** latency (s) to first open quadrant entry; **(D)** number of zone crossings. Number of mice per group (males (M)/females (F)): WT = 37 (17/20); KO = 43 (22/21). * $P < 0.05$, ** $P < 0.01$, vs WT. Data in Figs. 2–9 represent least-squares means $\pm$ S.E.M.

Fig. 3.

Locomotor activity (60 min). Number of successive photocell beam interruptions converted to distance (cm) traveled during the 60 min test session in 5-min intervals. **(A)** Distance traveled in the central zone. **(B)** Distance traveled in the peripheral zone. Inset: Distance traveled in the periphery averaged across all intervals. Number of mice per group (males/females): WT = 37 (17/20); KO = 44 (23/21). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, vs WT.

Fig. 4.

Novel object recognition (**A**) Percent time attending to novel object during the retention interval. (**B**) Percentage of mice that failed to attend to either object for at least 30 s during the retention interval (time limit = 10 min). Number of mice per group (males/females): WT = 37 (17/20); KO = 43 (23/21). * $P < 0.05$ vs WT.

Fig. 5.

Morris water maze, cued, test. (A) Day-1 latency (s) to the goal: mice were given six trials to reach the platform marked with an orange ball affixed to a 10 cm pole that protruded above the water with the start and goal unchanged on all six trials. (B) Days 2–6 latency (s) to the goal, two trials/day with the start and goal moved on each trial and the platform marked as on Day-1. Number of mice per group (males/females): WT = 36 (16/20); KO = 43 (23/21).

*P<0.05 vs WT.

Fig. 6.

Morris water maze, hidden platform, test. Cumulative distance from the starting point to the platform (cm) during the three phases of the test. (**A**) Acquisition, (**B**) reversal, and (**C**) shift. During reversal there was a significant genotype $\times$ day interaction ($P < 0.05$) in which KO mice improved their performance at a significantly different rate than WT mice. Number of mice per group (males/females): WT = 34–36 (14–16/20); KO = 43 (23/21).

Fig. 7.

Morris water maze, reversal, probe trial. Probe trials were given 24 h after the last platform trial of each phase of MWM testing. For platform-site crossovers, there were no significant effects on the acquisition or shift probe trials, but there was for reversal in which KO females crossed over the platform-site significantly more often than WT females. Numbers of mice per group were the same as that in Fig. 6. * $P < 0.05$ vs WT.

Fig. 8.

Locomotor activity before and after drug challenge with methamphetamine. Number of horizontal photobeam interruptions per 5-min interval. Mice were re-exposed to the test chambers for 30 min prior to drug administration in order to re-habituate them to the environment. They were then briefly removed, injected subcutaneously with 1.0 mg/kg of (+)-methamphetamine and returned to the test for an additional 2 h. Inset: Number of photobeam interruptions averaged across the 2-h post-challenge session. Number of mice per group (males/females): WT = 37 (17/20); KO = 42 (21/21). ** $P < 0.01$ vs WT.