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Glyoxalase 1 increases anxiety by reducing GABA$_A$ receptor agonist methylglyoxal

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Glyoxalase 1 (*Glo1*) expression has previously been associated with anxiety in mice; however, its role in anxiety is controversial, and the underlying mechanism is unknown. Here, we demonstrate that GLO1 increases anxiety by reducing levels of methylglyoxal (MG), a GABA$_A$ receptor agonist. Mice overexpressing *Glo1* on a Tg bacterial artificial chromosome displayed increased anxiety-like behavior and reduced brain MG concentrations. Treatment with low doses of MG reduced anxiety-like behavior, while higher doses caused locomotor depression, ataxia, and hypothermia, which are characteristic effects of GABA$_A$ receptor activation. Consistent with these data, we found that physiological concentrations of MG selectively activated GABA$_A$ receptors in primary neurons. These data indicate that GLO1 increases anxiety by reducing levels of MG, thereby decreasing GABA$_A$ receptor activation. More broadly, our findings potentially link metabolic state, neuronal inhibitory tone, and behavior. Finally, we demonstrated that pharmacological inhibition of GLO1 reduced anxiety, suggesting that GLO1 is a possible target for the treatment of anxiety disorders.

Introduction

Anxiety disorders, such as post-traumatic stress disorder, panic disorder, social phobia, specific phobia, and generalized anxiety disorder, comprise the most common psychiatric diseases in the United States (1). Mouse genetic studies have identified associations between glyoxalase 1 (*Glo1*) and anxiety-like behavior. Our previous work identified a copy number variant (CNV) in mice that causes a duplication of *Glo1* (2). The *Glo1* duplication was associated with increased *Glo1* expression and increased anxiety-like behavior (2). This CNV underlies differential *Glo1* expression and anxiety-like behavior previously reported among inbred mouse strains (3). Nevertheless, discrepant findings (4) have made the role of GLO1 in anxiety controversial (5). Here, we report the generation of mice with a Tg bacterial artificial chromosome (BAC) containing *Glo1*. We used these Tg mice to investigate the effect of *Glo1* on anxiety-like behavior. The BAC transgene allows overexpression of *Glo1* using the endogenous cis-regulatory elements on an isogenic background (6). BAC transgene expression is often copy-number dependent (7), providing an appropriate model of the *Glo1* CNV.

Importantly, the molecular mechanism underlying the effect of GLO1 on anxiety has yet to be identified. GLO1 is a ubiquitous cytosolic enzyme that catabolizes acyclic α-oxoaldehydes, particularly methylglyoxal (MG) (8). MG is mainly formed from the nonenzymatic degradation of the glycolytic intermediates dihydroxyacetone phosphate and glyceraldehyde-3-phosphate (9). In vitro studies have demonstrated a critical role for GLO1 in detoxifying MG: pharmacological inhibition of GLO1 results in MG accumulation (10), while overexpression of *Glo1* prevents MG accumulation (11). When MG accumulates to high levels, it has cytotoxic effects, including protein and nucleotide modification (advanced

glycation end products [AGEs]), generation of reactive oxygen species, and cell death (8, 12). Despite the well-known cellular effects of MG, there is no known mechanism linking MG to anxiety. In this study, we used BAC Tg mice to explore the mechanism by which GLO1 modulates anxiety. We focused on the possibility that GLO1 increases anxiety by regulating MG concentration in the brain. First, we established that low doses of MG are anxiolytic and that high doses of MG produce pharmacological effects similar to those of GABA$_A$ receptor agonists. We then assessed electrophysiological effects of MG on GABA$_A$ receptors in vitro.

Results

Glo1 copy number regulates Glo1 mRNA and protein expression. To investigate the effect of GLO1 on anxiety, we obtained a BAC containing the *Glo1* gene and its cis-regulatory elements (Figure 1A). The BAC contained partial copies of 2 additional genes, *Dnahc8* and *Btbd9*. We ablated the transcription start sites of these flanking genes using recombinogenic engineering (13) by inserting ampicillin and kanamycin cassettes into the first exons of each gene, respectively (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI61319DS1). Therefore, this BAC should only express *Glo1*. Using this modified BAC, we generated 3 lines of Tg mice on a C57BL/6J (B6) background. We measured BAC copy number in each line using quantitative real-time PCR (qPCR): Tg lines had 2, 8, and 10 copies of the BAC (Figure 1B). Tg animals were fertile, healthy, and did not display any grossly discernible physical or behavioral abnormalities (Supplemental Table 1).

Next, we measured *Glo1* mRNA expression using qPCR. Tg mice showed a copy number–dependent increase in *Glo1* mRNA in the brain (Figure 1C) and peripheral tissues (Supplemental Figure 2). Similarly, Tg mice displayed a copy number–dependent increase in GLO1 protein in the brain, as measured by immunoblot (Figure 1, D and E). Thus, the BAC dose-dependently increased *Glo1* mRNA and protein in Tg mice. Using gene-expression microarrays, we con-
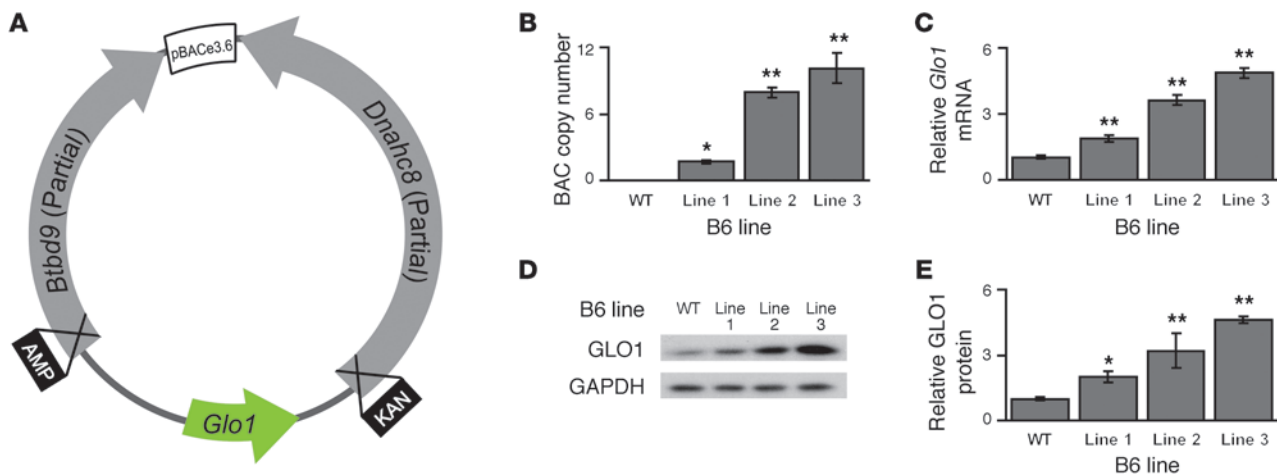
Figure 1
Glo1 copy number regulates *Glo1* expression. (**A**) The BAC (RP23-247F19) contained *Glo1* and partial copies of *Dnahc8* and *Btbd9*. The first exons of *Dnahc8* and *Btbd9* were ablated by inserting ampicillin (AMP) and kanamycin (KAN) cassettes, respectively. (**B**) BAC copy number was estimated by qPCR on genomic DNA ($n = 8$ WT and 3–5 Tg per line). $P < 10^{-10}$, 1-way ANOVA. (**C**) *Glo1* mRNA was estimated in whole brain by qPCR ($n = 12$ WT and 4–7 Tg per line). $P < 10^{-14}$, 1-way ANOVA. (**D** and **E**) GLO1 protein was estimated in whole brain by immunoblot ($n = 16$ WT and 3–6 Tg per line). $P < 10^{-11}$, 1-way ANOVA. Data are mean ± SEM. *$P < 0.05$; **$P < 0.0001$.

firmed that Tg mice overexpressed *Glo1*, but we did not observe any other changes in gene expression (Supplemental Table 2). In particular, we did not observe increased expression of *Dnahc8* or *Btbd9*, confirming successful ablation of their transcription from the BAC.

Glo1 overexpression increases anxiety-like behavior. We used male Tg mice to investigate the effect of *Glo1* overexpression on anxiety-like behavior in the open field (OF) test. The OF test is sensitive to anxiogenic and anxiolytic agents (14) and is associated with brain regions and neurotransmitters involved in human anxiety (15, 16). Further, the OF test was sensitive to differential *Glo1* expression in previous studies (2, 3). Tg mice displayed a significant, copy number–dependent decrease in time in the center of the OF (referred to throughout as center time) compared with that of WT mice (Figure 2A), reflecting increased anxiety-like behavior (17). WT and Tg mice did not significantly differ in total distance traveled during the test (Figure 2B). These data provide direct evidence that GLO1 increases anxiety-like behavior.

We generated 5 additional lines of Tg mice on a FVB/NJ (FVB) background to assess the generalizability of the anxiogenic effect of GLO1. FVB Tg mice showed increased *Glo1* copy number (Supplemental Figure 3A) and expression (Supplemental Figure 3B) compared with WT mice. In the OF test, FVB Tg mice displayed decreased center time compared with that of WT mice (Supplemental Figure 3C). These results replicate our findings in B6 Tg mice and demonstrate that GLO1 increases anxiety-like behavior in multiple genetic backgrounds.

In order to more thoroughly assess the role of GLO1 in anxiety, we tested WT and Tg mice in 2 additional well-established tests of anxiety-like behavior, the light-dark (LD) box test (18) and the elevated plus maze (19). In the LD box test, Tg mice spent less time in the light compartment compared with WT mice (Supplemental Table 3). WT and Tg mice did not significantly differ in the number of transitions between the 2 compartments, indicating normal locomotor activity (18, 20). In the elevated plus maze, Tg mice made fewer entries into the open arms compared with WT mice (Supplemental Table 4). WT and Tg mice did not differ in the number of total arm entries, again indicating normal locomotor activity. Together, these data demonstrate that *Glo1* overexpression increases anxiety-like behavior across different genetic backgrounds and multiple behavioral tests.

Glo1 increases anxiety by reducing MG levels. We used the B6 Tg line with the highest copy number (B6 line 3) to explore the molecular mechanism underlying GLO1's anxiogenic effect. Given known role of GLO1 in clearing MG (8), we hypothesized that GLO1 increases anxiety by regulating MG concentration. First, we measured MG metabolism in WT and Tg mice by a GLO1 enzymatic activity assay. Brain tissue from Tg mice metabolized a hemithioacetal substrate, formed from MG and glutathione, more rapidly than brain tissue from WT mice (Figure 3A). This reflects an increased capacity for clearing MG. We next measured MG concentration in the brains of WT and Tg mice by HPLC. Tg mice had an approximately 10% reduction of
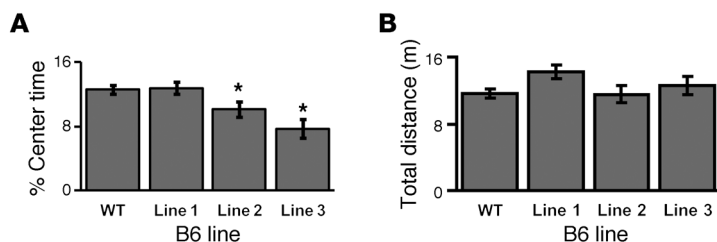

Figure 2
Glo1 overexpression increases anxiety-like behavior. WT and Tg littermates were tested in the OF test ($n = 74$ WT [pooled across all lines] and 14–21 Tg per line). (**A**) Center time. $P < 0.001$, 1-way ANOVA. (**B**) Total distance. $P > 0.1$, 1-way ANOVA. Data were pooled from multiple individual experiments. Data are mean ± SEM. *$P < 0.05$.
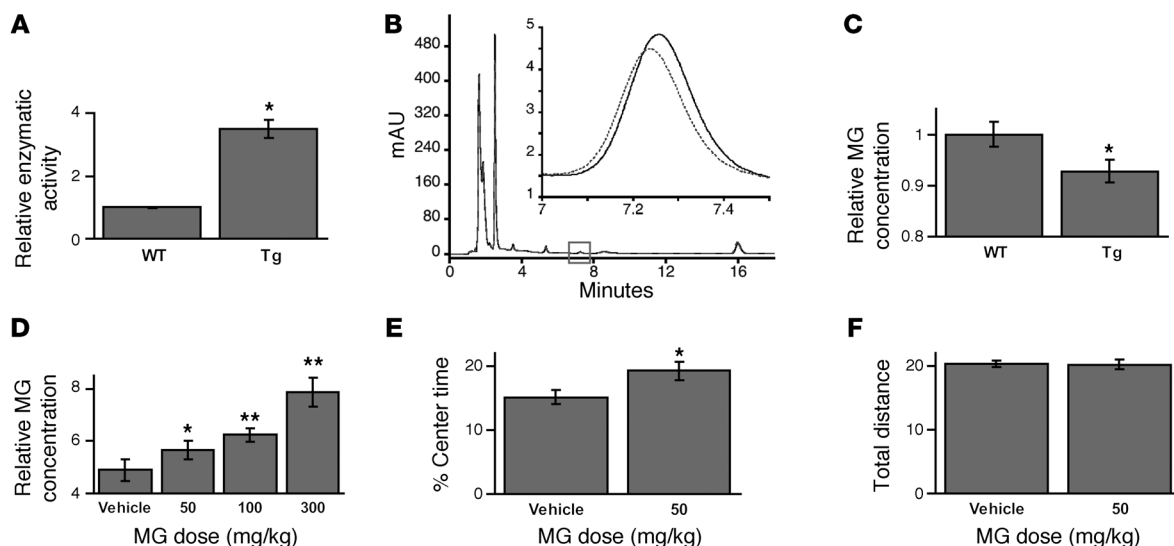
Figure 3
MG regulates anxiety-like behavior. (**A**) GLO1 enzymatic activity in whole brain (n = 3 WT and 3 Tg). (**B** and **C**) HPLC measurement of MG concentration in whole brain. (**B**) Representative chromatograms from WT (solid) and Tg (dashed) mice. An enlarged view of the relevant peak is shown in the inset. (**C**) Average MG concentration (n = 9 WT and 8 Tg). (**D**) HPLC measurement of MG concentration in whole brain after i.p. treatment with MG. Assay order had a significant effect on MG concentration and was used as a covariate in a 1-way analysis of covariance (ANCOVA) for the factor treatment (n = 4–7 per group). P = 0.009. (**E** and **F**) MG decreased anxiety-like behavior in the OF test. MG (50 mg/kg) (**E**) increased time in the center of the OF but (**F**) did not change total distance traveled (n = 18 per group). mAU, milli absorbance units. Data are mean ± SEM. *P < 0.05, **P < 0.0005.

MG concentration in the brain compared with WT mice (Figure 3, B and C). These results indicate that *Glo1* overexpression reduces MG concentration in the brain.

We hypothesized that MG is anxiolytic and that GLO1 increases anxiety by reducing endogenous MG concentration. To test this hypothesis, we administered MG i.p. to male WT mice and found that MG treatment dose-dependently increased MG concentration in the brain (Figure 3D and Supplemental Table 5). We then treated mice with MG (50 mg/kg) and tested them in the OF test 10 minutes after injection. MG treatment increased center time by approximately 30% (Figure 3E), without affecting distance traveled (Figure 3F).

We then used a 3-pronged approach to establish the validity and robustness of the anxiolytic effect of MG. First, we performed 6 replication studies to independently confirm the anxiolytic effect of MG in the OF test. A meta-analysis of the replication studies demonstrated that MG treatment significantly reduced anxiety-like behavior (Cohen's d = 0.78; meta-analysis

z-score = 5.0; P < 0.0001) (Supplemental Figure 4 and Supplemental Table 6). Second, we tested CD-1 mice treated with MG (50 mg/kg) in the OF test. Again, MG treatment increased center time in the OF, without affecting distance traveled (Supplemental Figure 5). This demonstrates that the anxiolytic effect of MG is not strain-specific. Third, we tested mice treated with MG (50 mg/kg) in the LD box test. MG treatment increased time spent in the light compartment, without affecting number of transitions (Supplemental Figure 6). This bolsters our initial finding and demonstrates that MG is anxiolytic across different behavioral tests of anxiety. Together, these results provide robust evidence that MG is acutely anxiolytic and that reduced MG levels mediate the anxiogenic effect of GLO1.

At high concentrations, MG has been shown to induce neuronal apoptosis (21). Therefore, we used in situ TUNEL staining to assess apoptosis in brains of mice treated with MG (Supplemental Figure 7). We found no evidence of increased apoptosis in mice treated with the anxiolytic dose of MG (50 mg/kg).
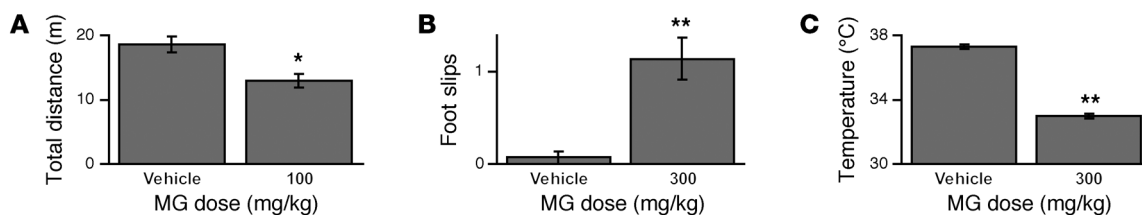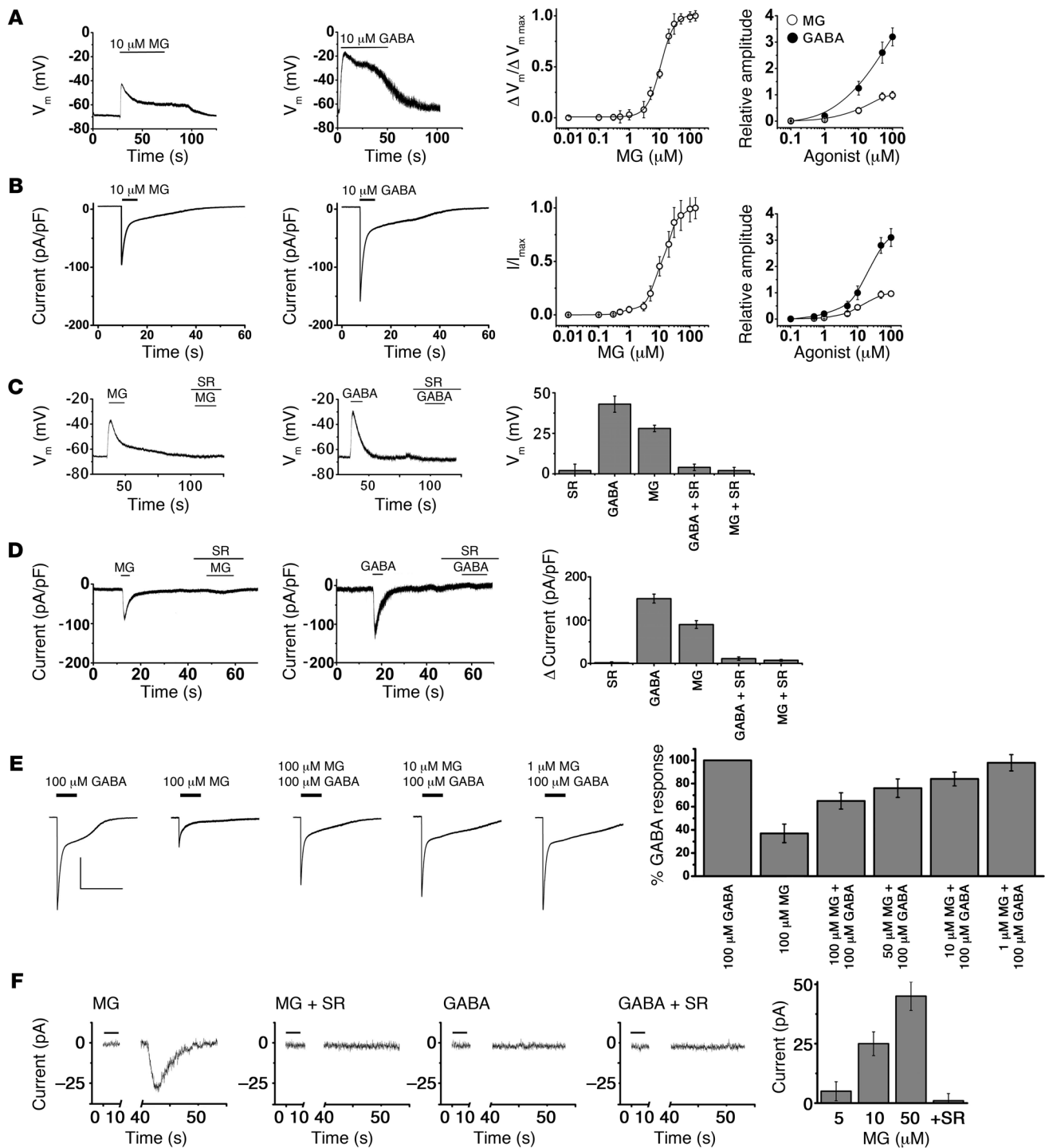

Figure 4
MG has GABAergic effects in vivo. (**A**) 100 mg/kg MG caused locomotor depression (n = 8 per group). (**B**) 300 mg/kg MG increased foot slips on the balance beam (n = 14 per group). Mann-Whitney U test, U = 26; P = 0.0002. (**C**) 300 mg/kg MG caused hypothermia (n = 10 per group). Data are mean ± SEM. *P < 0.05, **P < 0.0005.

Figure 5

MG is a GABA$_A$ receptor agonist in CGNs. (**A**) CGNs are depolarized by MG (white circles) or GABA (black circles) (EC$_{50}$, 10.5 ± 0.5 μM MG; Hill coefficient, 1.17). The relative amplitude of depolarization is shown normalized to the response of each cell to 100 μM MG. (**B**) MG evokes inward currents in a concentration-dependent manner (EC$_{50}$, 12 ± 0.7 μM; Hill coefficient, 1.13). The amplitude of currents is shown normalized to the peak response of each cell (I/Imax). (**C**) Depolarization evoked by 10 μM MG (left) or GABA (middle) was blocked by 10 μM SR. Mean data are plotted as a histogram (right). (**D**) Inward currents evoked by 10 μM MG (left) or GABA (middle) were also blocked by 10 μM SR. Mean data are plotted as a histogram (right). (**E**) Currents evoked by 100 μM GABA were reduced by coapplication of MG (left). Scale bars: 200 pA/pF, 25 s. Mean data are normalized to the current evoked by 100 μM GABA in each cell (right). (**F**) Currents observed approximately 40 seconds after the application of 10 μM MG to the inside of macropatches excised from CGNs were blocked when 10 μM SR was included in the pipette. Application of 10 μM GABA to the inside of macropatches did not evoke a current. Mean data are plotted as a histogram. The bar above each trace shows the duration of drug application. Data are mean ± SEM. n = 6–12 cells or macropatches per condition.

Notably, the time course for the anxiolytic effects of MG is distinct from the time course for its cytotoxic effects: MG is anxiolytic within minutes of administration, while high doses of MG require hours to induce apoptosis (21). Given the disparity in time course and the lack of apoptosis in the brains of mice treated with 50 mg/kg MG, we conclude that the anxiolytic effect of MG is independent of cytotoxicity.

MG has GABAergic properties in vivo. To further investigate the mechanism of MG's anxiolytic effect, we examined pharmacological properties of MG at higher doses. At 100 mg/kg, MG caused decreased locomotion (Figure 4A). At 300 mg/kg, MG caused ataxia (Figure 4B), hypothermia (Figure 4C), and sedation (data not shown). This behavioral profile is similar to those of known GABA$_A$ receptor agonists, such as ethanol, barbiturates, and benzodiazepines (22–24). GABA is the primary inhibitory neurotransmitter in the mammalian CNS and acts through 2 receptor subtypes, GABA$_A$ and GABA$_B$. The GABA$_A$ receptor subtype is a ligand-gated Cl$^-$ channel; when activated, it triggers increased intracellular Cl$^-$ concentration, membrane hyperpolarization, and reduced neuronal excitability (25). Positive modulators of GABA$_A$ receptors have anxiolytic properties (26) and are a mainstay of the clinical treatment of anxiety (27). Based on the pharmacodynamic profile of MG, we hypothesized that MG is a novel endogenous GABA$_A$ receptor agonist.

MG activates GABA$_A$ receptors. To test this hypothesis, we studied the electrophysiological effects of MG on primary cerebellar granule neurons (CGNs) using current-clamp recording to assess neuronal membrane potential (V_m) and voltage-clamp recording to measure ionic currents. In whole-cell current-clamp mode, extracellular application of MG rapidly depolarized CGNs in a concentration-dependent manner (Figure 5A). Initial, rapid depolarization was followed by repolarization of the V_m. In whole-cell voltage-clamp mode, application of MG produced inward currents at negative membrane potentials, characteristic of Cl$^-$ channel activation under our recording conditions (Figure 5B). The MG-evoked current diminished rapidly (Figure 5B), consistent with activation and desensitization of ligand-gated ion channels. These electrophysiological effects were similar to those elicited by application of GABA (Figure 5, A and B). Current density was concentration-dependent and similar in character to that evoked by GABA, while the relative amplitude was approximately one-third that of GABA. To establish that MG acts at GABA$_A$ receptors, responses were studied in the presence of the selective GABA$_A$ receptor antagonist SR-95531 (SR). SR inhibited more than 95% of the V_m (Figure 5C) and current-density changes (Figure 5D) induced by both MG and GABA.

We next established that electrophysiological effects of MG were specific to GABA$_A$ receptor activation. MG did not affect the major sodium currents (Supplemental Figure 8A) or potassium currents (Supplemental Figure 8, B and D–F) in CGNs. Moreover, MG did not activate GABA$_B$ receptors (Supplemental Figure 8C), glycine receptors (Supplemental Figure 8G), or glutamate receptors (Supplemental Figure 8, H and I). Together, these results demonstrate that activation of GABA$_A$ receptors is both necessary and sufficient for electrophysiological activity of MG.

We also compared the baseline electrophysiological properties of CGNs cultured from WT and Tg mice. Neurons from Tg mice were more excitable, displaying a diminished potassium leak current (I_{Kso}), a depolarized V_m, and increased cellular input resistance (Supplemental Figure 9).

MG is a competitive partial agonist at GABA$_A$ receptors. Next, we investigated whether MG acts competitively or noncompetitively with GABA. To do this, we coapplied MG and GABA to CGNs at 100 μM, concentrations that saturate GABA$_A$ receptor response (Figure 5B), indicating that binding sites are maximally occupied. Consistent with data shown in Figure 5B, 100 μM MG activated a current one-third the magnitude of that elicited by 100 μM GABA. In contrast, coapplication of 100 μM MG and 100 μM GABA activated a current approximately 60% of the magnitude elicited by 100 μM GABA alone (Figure 5E). The fraction of maximal current evoked by coapplication of MG and GABA increased as the concentration of MG was reduced (Figure 5E). These findings suggest that MG is a partial agonist at GABA$_A$ receptors and competes with GABA for the same binding site.

MG diffuses across the plasma membrane to activate GABA$_A$ receptors. Endogenous MG is generated intracellularly; therefore, we investigated intracellular effects of MG on GABA$_A$ receptors using macropatches of plasma membrane (PM). When applied to the intracellular face, MG elicited an inward current following a latency of approximately 40 seconds; this current was inhibited by SR applied to the extracellular face, suggesting that MG crosses the PM by diffusion and then activates GABA$_A$ receptors (Figure 5F). In contrast, GABA, which is unable to cross the PM, did not elicit a current when applied to the intracellular face of the PM macropatch (Figure 5F). These data suggest that endogenously produced MG accesses GABA$_A$ receptors by diffusing across the PM.

MG activates multiple GABA$_A$ receptor subtypes across multiple neuronal types. We next investigated the ubiquity of GABAergic effects of MG, specifically in different neuronal cell types and GABA$_A$ receptor subtypes. We demonstrated that electrophysiological effects of MG in CGNs were similar to those in primary cultures of hippocampal neurons (HNs) (Figure 6A), a cell type relevant to anxiety (28, 29). Specifically, MG dose-dependently evoked inward currents in HNs, with peak responses of approximately one-third of those evoked by GABA. Coapplication of 100 μM MG and 100 μM GABA activated a current of approximately 60% of the magnitude elicited by 100 μM GABA alone; the portion of the maximal current evoked by coapplication increased as the concentration of MG was reduced (Figure 6A). These data implicate MG as a partial GABA$_A$ receptor in HNs as well as CGNs. More importantly, they suggest that MG activates GABA$_A$ receptors to a similar degree in different neuronal cell types, including those relevant to anxiety.

We investigated the action of MG at specific GABA$_A$ receptor subtypes by co-applying 10 μM MG to HNs with subtype-selective positive modulators of GABA currents. MG-evoked currents were increased by coapplication of diazepam, midazolam, and zolpidem (Figure 6B). Diazepam, which acts at GABA$_A$ receptors containing α1, α2, α3, or α5 subunits, increased MG-evoked currents by approximately 60% and GABA-evoked currents by approximately 45%. Midazolam also modulates GABA$_A$ receptors containing α1, α2, α3, or α5 subunits in addition to γ2 subunits. Coapplication of 500 nM midazolam increased MG-evoked currents by approximately 40% and GABA-evoked currents by approximately 27%. Zolpidem selectively modules GABA$_A$ receptors containing α1 and γ2 subunits. Coapplication of 500 nM zolpidem increased MG-evoked currents by approximately 15% and GABA-evoked currents by approximately 17% (Figure 6B). Diazepam, midazolam, or zolpidem did not activate currents when
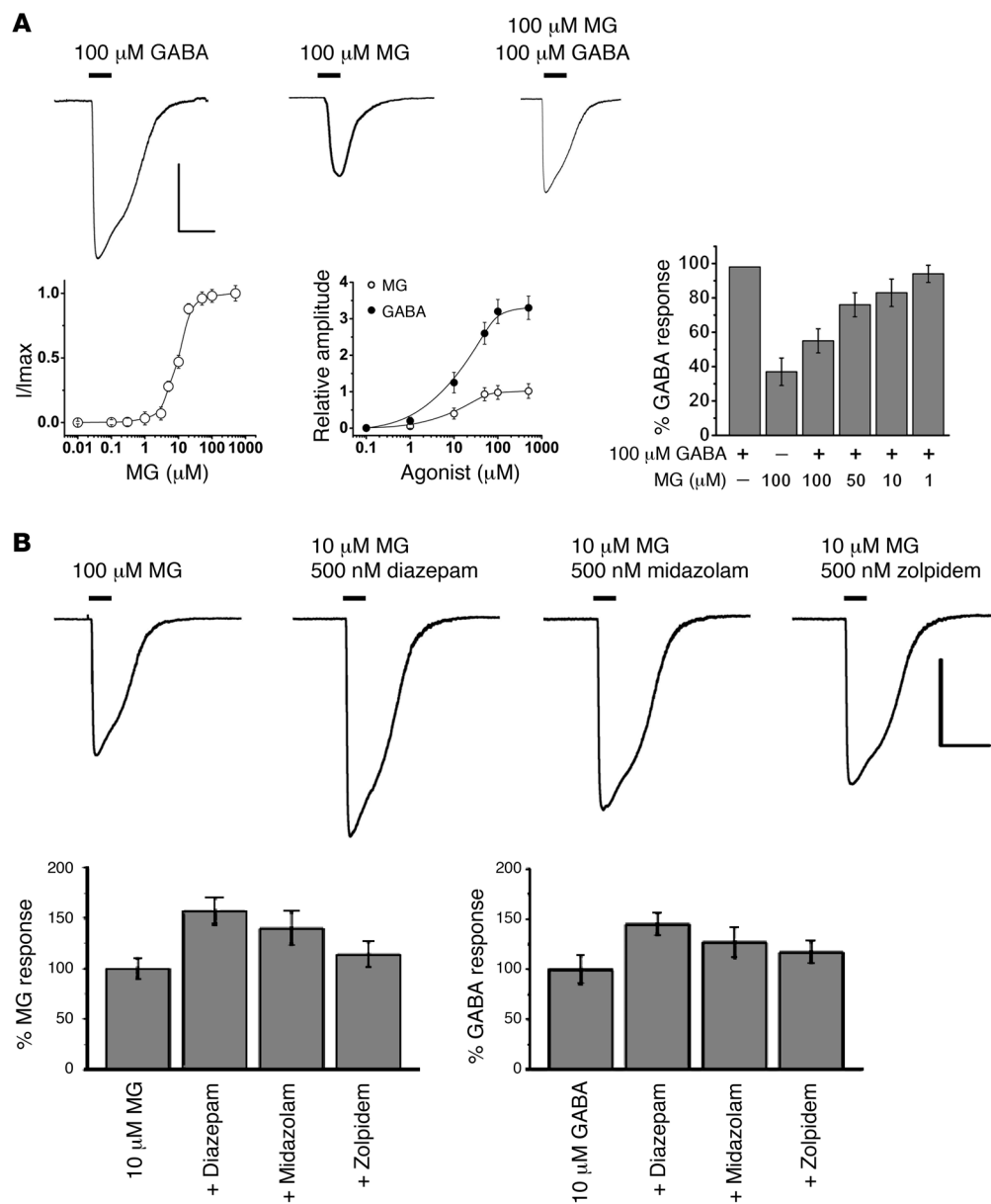
Figure 6
MG is a GABA$_A$ receptor agonist in HNs. (**A**) Both GABA and MG evoked concentration-dependent inward currents in HNs. The EC$_{50}$ of currents evoked by MG was 9.5 ± 0.9 μM, with a Hill coefficient of 1.05. The relative amplitude of the currents was normalized to that cell's response to 100 μM MG. Currents evoked by 100 μM GABA were reduced by coapplication of MG in a concentration-dependent manner. Scale bars: 1 nA, 10 s. Mean data for competition experiments are shown normalized to the current evoked by 100 μM GABA in each cell. (**B**) Currents evoked by 10 μM MG or GABA were augmented by coapplication of 500 nM diazepam, midazolam, or zolpidem. Scale bars: 1 nA, 10 s. Mean data are plotted as histograms. The bar above each trace shows the duration of drug application. Data are mean ± SEM for 8–10 cells per condition.

applied alone (data not shown), indicating negligible concentrations of endogenous MG and GABA in our experimental conditions. Taken together, these data demonstrate that MG operates a broad range of GABA$_A$ receptor subtypes, similar to GABA. Further, MG acts at GABA$_A$ receptors in HNs as well as CGNs, which have a diverse receptor population containing high levels of α1, α6, β2, β3, γ2, and δ subunits (30).

GLO1 inhibition reduces anxiety-like behavior. Finally, we evaluated the suitability of GLO1 as a target for anxiolytic drugs by synthesizing *S*-bromobenzylglutathione cyclopentyl diester (BrBzGCp2), a previously described GLO1 inhibitor (31, 32). We confirmed that BrBzGCp2 inhibited GLO1 enzymatic activity in vitro (Figure 7A). We then administered BrBzGCp2 (30 mg/kg) by i.p. injection to WT B6 mice and allowed MG levels to accumulate for 2 hours (ref. 10 and Figure 7B). GLO1 inhibition by BrBzGCp2 increased center time in the OF test

(Figure 7C), without changing distance traveled (Figure 7D). These data suggest that GLO1 inhibition increased MG concentration, thus reducing anxiety-like behavior. We obtained similar results in CD-1 mice treated with BrBzGCp2 (Supplemental Figure 10).

Finally, we investigated whether increasing MG concentration can rescue the anxiogenic effect of *Glo1* overexpression. In CD-1 mice, a naturally occurring *Glo1* duplication increases *Glo1* expression and anxiety-like behavior (ref. 2 and Supplemental Figure 11). Treatment with exogenous MG (50 mg/kg) reduced anxiety-like behavior in mice with the *Glo1* duplication (Supplemental Figure 11A), as did treatment with BrBzGCp2 (Supplemental Figure 11B). Together, these results indicate that increasing MG concentration reverses the anxiogenic effect of *Glo1* overexpression. Further, they illustrate the therapeutic potential of GLO1 inhibitors for the treatment of anxiety disorders.
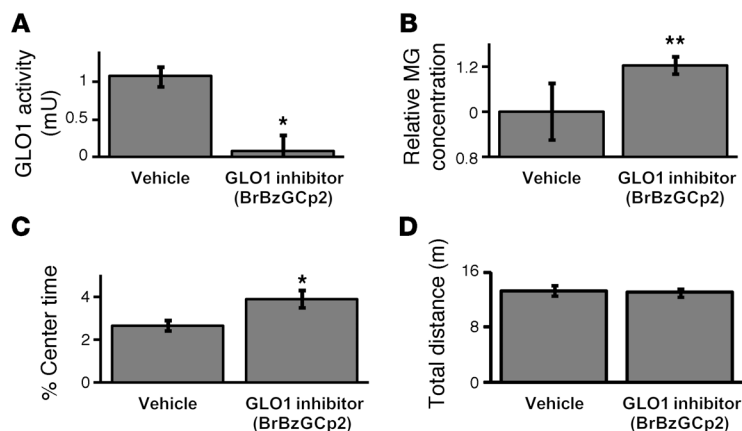
Figure 7
Pharmacological inhibition of GLO1 reduces anxiety-like behavior. (**A**) Enzymatic activity of 10 ng purified GLO1 protein treated with vehicle or 100 μM BrBzGCp2 (*n* = 3 vehicle and 4 BrBzGCp2). (**B**) HPLC measurement of MG concentration in whole brain 2 hours after i.p. treatment with vehicle or BrBzGSHCp2. Assay order had a significant effect on MG concentration and was used as a covariate in a 1-way ANCOVA for the factor treatment (*n* = 8 per group). $P < 0.0001$. (**C** and **D**) B6 mice were treated with i.p. vehicle or 30 mg/kg BrBzGCp2 and then tested in the OF test 2 hours after injection (*n* = 25 vehicle and 24 BrBzGCp2). (**C**) Center time. (**D**) Total distance. mU, milliunits (μmol/min). Data are mean ± SEM. *$P < 0.05$, **$P < 0.0001$.

Discussion

In the present study, we investigated the effect of GLO1 on anxiety and elucidated the underlying molecular mechanism. GLO1 had no obvious mechanistic relationship to anxiety. Rather, GLO1 was best characterized for detoxifying MG, a cytotoxic byproduct of glycolysis (8, 9). Using BAC Tg mice, we demonstrated that *Glo1* overexpression increased anxiety-like behavior. Tg mice had decreased MG levels in the brain, and administration of MG reduced anxiety-like behavior, suggesting that MG is an endogenous anxiolytic agent.

We next characterized MG as an endogenous partial agonist at GABA_A receptors, which are well-established mediators of anxiety (26). Physiologically relevant concentrations of MG elicited electrophysiological effects characteristic of GABA_A receptor activation in CGNs and HNs (Figures 5 and 6). MG-evoked currents were approximately one-third the magnitude of those elicited by GABA (Figure 5B and Figure 6A), and coapplication with MG reduced the magnitude of GABA-evoked currents (Figure 5E and Figure 6A). These data implicate MG as a partial competitive agonist at GABA_A receptors. While we did not identify the specific GABA_A receptor subtypes activated by MG, potent activation of currents in CGNs suggests that MG operates receptors containing α6 subunits, which are prevalent in CGNs (33). Further, MG-evoked currents were enhanced by coapplication of the benzodiazepines diazepam and midazolam as well as by zolpidem (Figure 6B). Diazepam and midazolam operate GABA_A receptors that contain α1, α2, α3, and α5 subunits, while zolpidem targets receptors with α1 and γ2 subunits. Taken together, these data suggest that MG acts at a broad range of GABA_A receptors, likely acting on neurons throughout the CNS. Future studies will be critical in further elucidating the pharmacological and biophysical responses of specific GABA_A receptor compositions to MG.

MG is generated intracellularly by both neurons and glia and then crossed the PM (Figure 5F) to activate GABA_A receptors in a paracrine manner. Thus, MG is expected to activate synaptic GABA_A receptors, which regulate phasic inhibitory signaling, as well as somatic, dendritic, and axonal GABA_A receptors, which contribute to tonic inhibitory signaling (34). Tonic inhibition via GABA_A receptors decreases cellular input resistance, which diminishes the propagation of excitatory currents and decreases the probability that an action potential will be initiated by excitatory inputs (35). Many therapeutic agents, including those for anxiety disorders, target extrasynaptic GABA_A

receptors with the aim of modulating tonic inhibition (36). We speculate that the actions of MG at extrasynaptic GABA_A receptors mediate tonic inhibition and anxiety. Indeed, midazolam, which is known to increase tonic conductances (37), enhanced MG-evoked currents (Figure 6B).

The coordinated release of synaptic vesicles ensures that GABA concentrations in the synaptic cleft peak in the millimolar range (38). In contrast, concentrations of GABA in the extracellular space are in the submicromolar range (39). The concentration of MG in the brain was approximately 5 μM (Supplemental Table 5), reflecting concentrations both at the synaptic cleft and in the extracellular space. Therefore, future studies must directly measure the effect of MG on extrasynaptic receptors in order to determine the relative contribution of MG to phasic and tonic inhibitory activity in the CNS. In particular, measuring inhibitory postsynaptic currents in slice preparations may further elucidate the role of MG in neural physiology.

We provided evidence that physiological concentrations of MG produce robust electrophysiological effects in vitro. A concentration of 5 μM MG was on the steep segment of the concentration-response curve, at which a small change in concentration elicits large changes in current and membrane potential (Figure 5, A and B, and Figure 6A). Accordingly, we showed that small changes in MG concentration in vivo substantially altered behavior. Tg mice exhibited less than a 10% decrease in MG concentration (Figure 3C) but exhibited a marked anxiety-like phenotype, spending up to 30% less time in the center of the OF than did WT mice (Figure 2A). Similarly, exogenous administration of MG (50 mg/kg) increased MG concentration by only 15% (Figure 3D and Supplemental Table 5) but increased center time in the OF by almost 30% (Figure 3E). Thus, GABA_A receptors and downstream behaviors are exquisitely sensitive to relatively small changes in MG concentration, consistent with an important role for MG under normal physiological conditions. Finally, because MG levels increase under conditions of high metabolic load (8, 40), our findings suggest a mechanism whereby metabolic state regulates neuronal inhibitory tone and behavior.

To our knowledge, activation of GABA_A receptors represents a novel physiological role for MG. Previous studies have established the role of MG in AGE formation and cytotoxicity as well as diseases in which these effects are relevant to pathogenesis, such as diabetic complications, cancer, and aging (40–44). Because supraphysiological levels of MG (100–1,000 μM) induce

AGEs, reactive oxygen species, and apoptosis in neurons (21, 45, 46), these effects have been thought to underlie the role of MG in CNS disorders, including anxiety. For instance, chronic intracerebroventricular administration of MG was reported to reduce anxiety-like behavior, coincident with AGE accumulation (47). In contrast, our data demonstrate that physiological levels of MG have a selective effect on GABA$_A$ receptors and elicit behavioral effects within minutes, suggesting a novel role for MG in the CNS that is independent of cytotoxicity.

Our findings put the previous controversy regarding the role of GLO1 in anxiety-like behavior into context. Hovatta et al. reported a positive correlation between *Glo1* expression and anxiety-like behavior among inbred mice and proposed a causal role for GLO1 in anxiety (3). Our previous work identified a CNV in mice that causes a duplication of *Glo1* and was associated with increased *Glo1* expression and anxiety-like behavior (2). This CNV represents the molecular polymorphism likely responsible for the prevalence of differential *Glo1* expression among inbred mice. However, discrepant findings have cast doubt on the association between GLO1 and increased anxiety. In particular, Kromer et al. generated 2 inbred mouse lines selected for high and low anxiety-like behavior; the low anxiety line showed greater *Glo1* expression than the high anxiety line (4). Results from these selected lines are directionally opposite of results from Hovatta et al. and Williams et al. (2, 3). In this study, we generated BAC Tg mice with increased copies of *Glo1* to model the naturally occurring CNV. To isolate the effect of *Glo1*, Tg mice overexpressed *Glo1* but not the other 3 genes in the CNV. Using these Tg mice, we provided direct evidence that *Glo1* copy number regulates *Glo1* expression and anxiety. This effect was strain-independent, as *Glo1* was anxiogenic on both B6 and FVB backgrounds. We note that, of the 3 B6 Tg lines, only the 2 with the highest *Glo1* expression exhibited increased anxiety-like behavior (Figure 2A). FVB Tg lines showed a similar pattern (Supplemental Figure 3C), suggesting that the anxiogenic effect of GLO1 is dose-dependent. Taken together, our data indicate that increased *Glo1* copy number increases *Glo1* expression and anxiety-like behavior. This study helps resolve the apparent discrepancy in the literature: it appears that the bidirectionally selected and subsequently inbred lines of Kromer et al. (4) differentially fixed the *Glo1* CNV. The low anxiety line carried the duplication, while the high anxiety line did not (47). The difference in *Glo1* expression is likely offset by numerous other alleles that collectively underlie the response to selection. Our data unequivocally support the hypothesis that increased *Glo1* expression causes increased anxiety-like behavior and are strengthened by the use of isogenic backgrounds.

The present study may also provide mechanistic insight into GLO1's proposed associations with other CNS diseases, such as autism (48, 49), affective disorders (50, 51), panic disorder (52), and schizophrenia (53). Human genome-wide association studies have identified an association between restless legs syndrome (RLS) and a haplotype containing *GLO1* (54, 55). Our results suggest that GLO1's role in GABAergic signaling could underlie its association with RLS. Indeed, GABA$_A$ receptor agonists are used to treat RLS (56). Finally, we established that GLO1 is a target for the treatment of anxiety disorders and demonstrated the anxiolytic effect of a GLO1 inhibitor. Similar drugs may be useful in the treatment of anxiety and other diseases linked to GABA signaling, including epilepsy and sleep disorders (57, 58).

Methods

Detailed methods are provided in the Supplemental Methods.

Animals. Behavioral tests used 7- to 10-week-old male mice. For pharmacology experiments, B6 mice were obtained from The Jackson Laboratory. For studies with Tg mice, WT and Tg littermates were tested in parallel. WT mice from each line did not significantly differ and were pooled.

BAC Tg mice. The mouse BAC RP23-247F19 was obtained from the Children's Hospital Oakland Research Institute and modified by RED/ET recombination (13). The modified BAC was purified and injected into B6 or FVB pronuclei by the University of Chicago's Transgenic Core Facility. Accordingly, Tg founders were derived on pure B6 or FVB backgrounds.

BAC copy number. Genomic DNA was used in qPCR with SYBR reagents (Applied Biosystems). Primers targeted *Glo1* and a region of *Btbd9* outside the BAC region.

mRNA expression. *Glo1* mRNA was measured as previously described by qPCR with SYBR reagents (Applied Biosystems) (2). *Glo1* expression was normalized to *Actb* and reported as fold change versus WT (59).

Microarray data. Microarray data are available from GEO (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE36819.

Immunoblot. Twenty µg of total protein was separated by SDS-PAGE. Membranes were probed with primary antibodies against Glo1 (gift from Iiris Hovatta, University of Helsinki, Helsinki, Finland) and GAPDH (Cell Signaling Technology). Peroxidase-conjugated secondary antibodies were then used (Jackson ImmunoResearch Laboratories Inc.). Blots were developed with Pierce ECL Plus (Thermo Fisher Scientific), digitized, and band intensity was measured by densitometry.

OF test. The OF test was administered as previously described (2). The center size was 18 × 18 cm.

Enzymatic activity. Glo1 activity was assayed by measuring the rate of formation of S-D-lactoylglutathione as previously reported (60).

HPLC. MG concentration was measured by HPLC as previously reported (61), with modifications.

MG pharmacology. MG was obtained from Sigma-Aldrich, filter-sterilized (0.22-µm filter; Millipore), diluted in vehicle (0.9% NaCl), and adjusted to a pH of approximately 7.4. Mice were injected i.p. with 10 ml/kg MG or vehicle.

Electrophysiology. Recordings were performed in primary cultures of CGNs or HNs after 6 to 9 days in culture. HNs were a gift from Suzanne Paradis (Brandeis University). Whole-cell patch-clamp analysis was performed as previously described (62). Macropatch recording was performed with 1.5 to 2 MΩ electrodes filled with bath solution. The inside of excised patches was perfused with electrode solution containing the indicated reagents.

Glo1 inhibitor. BrBzGCp2 was prepared as previously described (31, 32), with modifications. BrBzGCp2 was dissolved in vehicle (8% DMSO and 18% Tween-80). Mice were treated i.p. with 10 ml/kg BrBzGCp2 or vehicle.

Statistics. All statistical analyses were carried out using StatView for Windows (SAS Institute Inc.) unless otherwise noted. Normally distributed data were analyzed by *t* tests or ANOVA, as appropriate. The *t* tests were 2-tailed unless otherwise noted. Post-hoc tests were used to determine significant differences between groups when the ANOVA yielded a significant result. For data that were not normally distributed, the Mann-Whitney *U* test was used. For the meta-analysis of the anxiolytic effect of MG, results from individual experiments were compiled and analyzed with MIX 1.7 software using a fixed-effect model (63). For all tests, $P < 0.05$ was considered statistically significant. The figure legends indicate the specific tests used and their respective *P* values. For statistical analysis of the microarray data, *P* values were converted to *q* values using Q value software (64), and $q < 0.05$ was considered statistically significant.

Study approval. All procedures involving mice were approved by the University of Chicago's Institutional Animal Care and Use Committee.

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