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Neuronal overexpression of *Glo1* or amygdalar microinjection of methylglyoxal is sufficient to regulate anxiety-like behavior in mice

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Abstract

GLO1 (Glyoxalase1) is a ubiquitous cellular enzyme that detoxifies methylglyoxal (MG), which is a byproduct of glycolysis. Previously, we showed that ubiquitous overexpression of *Glo1* reduced concentrations of MG and increased anxiety-like behavior, whereas systemic injection of MG reduced anxiety-like behavior. We further showed that MG is a competitive partial agonist at GABA-A receptors. Based on those data we hypothesized that modulation of GABAergic signaling by MG underlies *Glo1* and MG's effects on anxiety-like behavior.

As previous studies used ubiquitous overexpression, we sought to determine whether neuronal *Glo1* overexpression was sufficient to increase anxiety-like behavior. We generated ROSA26 knock-in mice with a floxed-stop codon upstream from human *Glo1* (FLOXGlo1KI) and bred them with mice expressing CRE recombinase under the direction of the *Synapsin 1* promoter (Syn-CRE) to limit overexpression of *Glo1* specifically to neurons.

Furthermore, since previous administration of MG had been systemic, we sought to determine if direct microinjection of MG into the basolateral amygdala (BLA) was sufficient to reduce anxiety-like behavior. Thus, we performed bilateral microinjections of saline, MG (12μM or 24μM), or the positive control midazolam (4mM) directly into the BLA.

FLOXGlo1KIxSyn-CRE mice showed significantly increased anxiety-like behavior compared to their FLOXGLO1xWT littermates. In addition, bilateral microinjection of MG and midazolam

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significantly decreased anxiety-like behavior compared to saline treated mice. These studies suggest that anatomically specific manipulations of *Glo1* and MG are sufficient to induce changes in anxiety-like behavior.

Keywords

Mounting evidence supports a role for *Gyloalase 1 (Glo1)* and its substrate methylglyoxal (MG) in the regulation of anxiety-like behavior [1–4]. GLO1 is a ubiquitous cytosolic enzyme primarily responsible for catalyzing the reaction between glutathione and acyclic α -oxoaldehydes; particularly, MG [5]. MG is a byproduct of glycolysis that is mainly formed from the nonenzymatic degradation of the glycolytic intermediates dihydroxyacetone phosphate and glyceraldehyde-3-phosphate [5]. GLO1 has a critical role in the clearance of MG with overexpression of *Glo1* preventing MG accumulation and GLO1 inhibition resulting in MG accumulation [3,5].

We previously demonstrated that a duplication of a region containing 4 genes that included *Glo1* was associated with increased *Glo1* mRNA and increased anxiety-like behavior [2]. Additionally, we found that transgenic mice ubiquitously overexpressing *Glo1* alone showed a copy-number-dependent increase in anxiety-like behavior [3]. Conversely, acute administration of MG or a GLO1 inhibitor, S-bromobenzylglutathione cyclopentyl diester (pBBG), decreased anxiety-like behavior in wild-type animals [3]. Electrophysiological recordings indicated that MG was a competitive partial agonist at GABA-A receptors and that MG is able to activate these receptors through diffusion across the cell membrane at physiologically relevant concentrations [3]. Based on these data we hypothesized that the action of MG at GABA-A receptors likely contributes to its anxiolytic effects.

Many studies have implicated the basolateral amygdala (BLA) in both normal and pathological anxiety [6–9]. Neuroimaging studies have reported differences in amygdala-prefrontal circuitry in patients with anxiety disorders [10]. Additionally, direct injection of midazolam, a positive allosteric modulator at GABA-A receptors (benzodiazepine), into the BLA reduces anxiety-like behavior in mice [11]. However, because *Glo1* expression and MG production occur in all tissues and all brain regions, the role of the BLA in mediating the effects of MG on anxiety-like behavior have not been explored.

The studies performed here aimed to determine whether the effects of *Glo1*/MG on anxiety-like behavior are peripherally or centrally mediated and if central, to determine whether the BLA was sufficient for the anxiolytic effects of MG. All studies used male mice that were group housed on a standard light cycle (12L/12D) and given unlimited access to standard food and water. Data were analyzed using Student's *t*-test or ANOVA as appropriate. Holm-Sidak multiple comparisons procedures were used to determine which treatments yielded significantly different responses. *p*-values < 0.05 were considered significant.

In the first experiment, tissue-specific overexpression of *Glo1* was achieved on a C57BL/6J (B6) background by knock-in of human *Glo1* with an upstream floxed STOP to the ROSA26 locus (Fig.1A; FLOXGlo1KI; Albert Einstein College of Medicine and Ozgene

Pty Ltd (Bentley WA, Australia)). Insertion of the FLOXGlo1KI construct was confirmed by genotyping DNA from mice using the following primers: Fwd: ACTGAAGATGATGCGACCCAG; Rev: CACCTGTTCAATCCCCCTGC. Mice homozygous for FLOXGlo1KI were bred at The University of Chicago to hemizygous mice expressing CRE recombinase under the direction of the *synapsin 1* promoter (Syn-CRE; B6.Cg-Tg(Syn1-cre)671Jxm/J, obtained from The Jackson Laboratory; generated on B6;CBAF1 background, founders bred to C57BL/6NHsd) which is only expressed in neurons. Thus, in behavioral studies we used littermates (8-13 weeks old; from 11 litters total) that were always positive for FLOXGlo1KI, but were either CRE positive (overexpress *Glo1*; n=26) or CRE negative (not overexpress *Glo1*; n=19). These mice allowed us to assess the impact of over-expressing *Glo1* only in neurons on anxiety-like behavior in the open-field test (OFT).

No deficits were seen in the general health for either FLOXGlo1KI mice or FLOXGlo1KIxSyn-CRE mice. For example, there was no effect of genotype on weight in these mice ($p=0.889$ by Two-tailed *t*-Test). To confirm overexpression of *Glo1* in the brain, we performed western blots as previously described [3]. Blots were probed using antibodies against GLO1 (Santa Cruz Biotechnology; sc-67351) and α -tubulin (Cell Signaling Technology; #2125). Blots were developed using Pierce ECL Plus (Thermo Fisher Scientific), digitized and band intensity was measured using ImageJ (NIH; <http://rsbweb.nih.gov/ij/>). Western blots suggested FLOXGlo1KIxSyn-CRE mice (n=3) have increased GLO1 compared to WT littermates (n=4), although this difference was suggestive (Fig. 1B; $t(1,5)=1.955$, $p=0.054$ by One-tailed *t*-Test) rather than significant.

To further confirm overexpression of *Glo1*, we assayed GLO1 enzymatic activity by measuring the rate of formation of S-D-lactoylglutathione as previously described [3]. Briefly, brain (n=8 per group) or liver (n=4 per group) homogenate (50 μ g protein) was added to a hemithioacetal substrate (incubate 2 mM MG and 2 mM Glutathione at 37°C for 10 minutes), and the absorbance at 240 nm was measured every 30 seconds for 4 minutes. FLOXGlo1KIxSyn-CRE mice showed significantly increased GLO1 enzymatic activity in the brain compared to their FLOXGlo1KI $\times$ WT littermates (Fig. 1C; $F(1,15)=5.823$; $p<0.05$). There was also an effect of cohort for brain enzymatic activity ($F(1,15)=21.845$; $p<0.001$), likely due to the extended freeze time (~1 year) of samples from cohort 1 in comparison to those of cohort 2 (~4 hrs frozen; 4 mice per cohort). However, there was no significant cohort $\times$ genotype interaction ($F(1,15)=0.506$; $p=0.491$). There was also no difference in GLO1 enzymatic activity in the liver between FLOXGlo1KIxSyn-CRE and their FLOXGlo1KI $\times$ WT littermates (Fig. 1D; Two-tailed *t*-test, $p=0.605$). Thus, FLOXGlo1KIxSyn-CRE mice showed about a ~30% increase in GLO1 enzymatic activity that was limited to the brain (central; Fig. 1C), but not liver (periphery; Fig. D).

To assess anxiety-like behavior in the OFT, mice were placed into chambers (AccuScan, Columbus, OH, USA) surrounded by infrared detection beams on the X, Y and Z-axes which tracked the animals' activity. Locomotor activity and center duration were assessed using automated Versamax software. Chambers measured 43 $\times$ 43 $\times$ 33 cm (width $\times$ depth $\times$ height) and had dim overhead fluorescent lighting (14 lux). Center size was 26 $\times$ 26 cm. We found that FLOXGlo1KIxSyn-CRE spent significantly less time than their FLOXGlo1KI $\times$

WT littermates in the center during the first 5 minutes (Fig.1E, Two-tailed *t*-test, $p<0.01$). Importantly, there was no difference in total distance traveled (Fig.1F; Two-tailed *t*-test, $p=0.729$) suggesting that differences in anxiety-like behavior are not due to changes in overall activity.

Prior studies with ubiquitously overexpressed *Glo1* indicated that higher levels of GLO1 activity were needed to induce behavioral changes [3]. The somewhat modest increase in GLO1 protein and enzymatic activity observed in this study (~20-30%) may reflect our use of whole brain homogenate, which includes other cell types (e.g. glia) that do not overexpress GLO1 and thus dilute the increased protein and enzymatic activity induced in neurons. Regardless of the reason for the modest increase in protein and enzymatic activity observed in this study, these levels were sufficient to alter anxiety-like behavior in the OFT.

In a second experiment intended to further assess neuroanatomical specificity, we implanted bilateral cannula directed to the BLA. The BLA has long been shown to be involved in the regulation of anxiety-like behavior [8,9,11–13]. Because MG is a GABA-A receptor agonist, we hypothesized that direct injection of MG into the BLA would reduce anxiety-like behavior in the OFT. Thus, we performed bilateral microinjections of vehicle, MG (12 μ M or 24 μ M) or midazolam, a benzodiazepine, as a positive control [11] directly into the BLA (Fig.2A) and then measured anxiety-like behavior in the OFT.

In the microinjection study, B6 mice were purchased from the Jackson Laboratory (7-9 weeks old; JAX). Mice were anesthetized with ketamine/xylazine (88/1.3 mg/kg I.P.; Sigma-Aldrich) and bilaterally implanted with guide cannula targeting the BLA (AP -1.4, ML $\pm$ 3.2, DV 5.1). Animals were allowed to recover for 5-6 days. On test day, injection cannulas were inserted into guide cannula; the injection cannula extended 1 mm past the guide cannula. Vehicle (0.9% saline; n=15), 4 mM midazolam (positive control, n=15; UC429 Sigma-Aldrich), 12 μ M MG (n=15; M0252 Sigma-Aldrich), or 24 μ M MG (n=9) was bilaterally microinjected at a constant flow rate of 0.25 μ l/min for 2 minutes (0.5 μ l total) with an additional 2 minutes allowed for diffusion. Following microinjection, mice were placed directly into the OFT. There was a significant main effect of treatment on center duration in the OFT over 30 minutes (Fig.2B; $F(3, 45)=13.765$; $p<0.001$). Individual post-hoc tests revealed increases in center duration compared to vehicle for all treatments (MG 12 μ M, $p<0.05$; MG 24 μ M, $p<0.01$ and midazolam, $p<0.01$ by Holm-Sidak post-hoc comparisons). Importantly, there was no effect of treatment on total distance traveled (Fig. 2C; $F(3,45)=0.887$; $p=0.456$). Differences in center duration and distance traveled between the VEH treated mice within this study and the FLOX*Glo1*KI $\times$ WT mice in the previous study are likely due to the increased stress associated with microinjection directly before testing.

Overall, these data suggest *Glo1*'s effects on anxiety-like behavior are centrally mediated as overexpression of *Glo1* in neurons was sufficient to increase anxiety-like behavior. They also suggest that MG is able to modulate anxiety-like behavior in the OFT through direct application into the BLA as there was a dose dependent increase in center duration after direct injection of MG into the BLA that was comparable to that of midazolam. Importantly,

the doses of MG used (12 μ M and 24 μ M) are within a physiologically relevant range based on previous reports of MG concentration in the brain [3,4].

These data are consistent with previous studies suggesting that changes in *Glo1* expression or MG concentration in the brain are sufficient to regulate anxiety-like behavior in mice. Hovatta et al. (2005) found that lentiviral mediated overexpression of *Glo1* in the anterior cingulate cortex increased anxiety-like behavior and lentiviral mediated knockdown of *Glo1* in the anterior cingulate cortex reduced anxiety-like behavior in 129S6/SvEvTac mice. Hovatta et al. (2005) also found that lentiviral knockdown of *Glo1* in the anterior cingulate cortex reduced anxiety-like behavior in B6 mice, however, lentiviral mediated overexpression of *Glo1* in the anterior cingulate cortex did not increase anxiety-like behavior in B6 mice for reasons that were not entirely clear. In the present study, FLOXGlo1KI $\times$ SynCRE mice on a B6 background, which overexpress *Glo1* in all neurons showed increased anxiety-like behavior. These results show that neuronal over expression has a greater effect on anxiety-like behavior than lentiviral mediated overexpression that is limited to the anterior cingulate cortex, at least when compared on a B6 background.

Hambusch et al. (2010) previously found that i.c.v. administration of MG for 6 days reduced anxiety-like behavior in the elevated plus maze. The studies presented here build on those of Hambusch et al. (2010) by administering MG to the BLA, which is strongly associated with anxiety-like behavior [8,9,11–13]. Taken together with prior studies, our data suggest that *Glo1* regulates anxiety-like behavior through neurocircuitry typically associated with anxiety-like behavior.

Our data also support the therapeutic potential of modulating MG levels for the treatment of anxiety disorders. MG accumulation is fundamentally different from treatment with currently used anxiolytic drugs, such as benzodiazepines, because MG is an endogenously produced competitive partial agonist, rather than a positive modulator, such as midazolam [3,12]. As MG can diffuse across the cell membrane to act on GABA-A receptors, MG may preferentially act at extrasynaptic GABA-A receptors where the concentration of GABA is low [14,15]. At high levels, MG is cytotoxic[5]. Thus, an alternative and perhaps more promising therapeutic strategies is to raise MG levels by inhibiting the GLO1[16,17]. Application of a GLO1 inhibitor is expected to potentiate the activity of GABA-A receptors by reducing the degradation of MG [15]. As MG production increases with increased metabolic load [18,19], this strategy may lead to more region specific increases in MG with treatment. Thus, treatments that increase MG concentrations may have qualitatively different effects as compared to existing approaches.

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Abbreviations

(GLO1)	Glyoxalase1
(MG)	Methylglyoxal
(OFT)	open-field test
(BLA)	basolateral amygdala
(pBBG)	S-bromobenzylglutathione cyclopentyl diester

References

- [1]. Hovatta I, Tennant RS, Helton R, Marr R. a, Singer O, Redwine JM, et al. Glyoxalase 1 and glutathione reductase 1 regulate anxiety in mice. *Nature*. 2005; 438:662–6. [PubMed: 16244648]
- [2]. Williams R, Lim JE, Harr B, Wing C, Walters R, Distler MG, et al. A common and unstable copy number variant is associated with differences in Glo1 expression and anxiety-like behavior. *PLoS One*. 2009; 4:e4649. [PubMed: 19266052]
- [3]. Distler MG, Plant LD, Sokoloff G, Hawk AJ, Aneas I, Wuenschell GE, et al. Glyoxalase 1 increases anxiety by reducing GABA A receptor agonist methylglyoxal. *J. Clin. Invest.* 2012; 122:2306–2315. [PubMed: 22585572]
- [4]. Hamsch B, Chen B-GG, Brenndörfer J, Meyer M, Avrabos C, Maccarrone G, et al. Methylglyoxal-mediated anxiolysis involves increased protein modification and elevated expression of glyoxalase 1 in the brain. *J. Neurochem.* 2010; 113:1240–51. [PubMed: 20345757]
- [5]. Thornalley PJ. Pharmacology of methylglyoxal: formation, modification of proteins and nucleic acids, and enzymatic detoxification—a role in pathogenesis and antiproliferative chemotherapy. *Gen. Pharmacol.* 1996; 27:565–73. [PubMed: 8853285]
- [6]. Earnheart JC, Schweizer C, Crestani F, Iwasato T, Itohara S, Mohler H, et al. GABAergic control of adult hippocampal neurogenesis in relation to behavior indicative of trait anxiety and depression states. *J. Neurosci.* 2007; 27:3845–54. [PubMed: 17409249]
- [7]. Davis M. The role of the amygdala in fear and anxiety. *Annu. Rev. Neurosci.* 1992; 15:353–375. [PubMed: 1575447]
- [8]. Tye KM, Deisseroth K. Optogenetic investigation of neural circuits underlying brain disease in animal models. *Nat. Rev. Neurosci.* 2012; 13:251–66. [PubMed: 22430017]
- [9]. Janak PH, Tye KM. From circuits to behaviour in the amygdala. *Nature*. 2015; 517:284–292. [PubMed: 25592533]
- [10]. Möhler H. The GABA system in anxiety and depression and its therapeutic potential. *Neuropharmacology*. 2012; 62:42–53. [PubMed: 21889518]
- [11]. Heldt S, Ressler K. Localized injections of midazolam into the amygdala and hippocampus induce differential changes in anxiolytic-like motor activity in mice. *Behav. Pharmacol.* 2006; 17:349–356. [PubMed: 16914953]
- [12]. Kent JM, Mathew SJ, Gorman JM. Molecular targets in the treatment of anxiety. *Biol. Psychiatry*. 2002; 52:1008–30. [PubMed: 12437941]
- [13]. Vyas A, Chattarji S. Modulation of different states of anxiety-like behavior by chronic stress. *Behav. Neurosci.* 2004; 118:1450–4. [PubMed: 15598155]
- [14]. Vithlani M, Terunuma M, Moss SJ. The dynamic modulation of GABA(A) receptor trafficking and its role in regulating the plasticity of inhibitory synapses. *Physiol. Rev.* 2011; 91:1009–22. [PubMed: 21742794]
- [15]. Distler MG, Plant LD, Sokoloff G, Hawk AJ, Aneas I, Wuenschell GE, et al. Glyoxalase 1 increases anxiety by reducing GABAA receptor agonist methylglyoxal. *J. Clin. Invest.* 2012; 122:2306–15. [PubMed: 22585572]

- [16]. McMurray KMJ, Distler MG, Sidhu PS, Cook JM, Arnold L. a, Palmer A. a, et al. Glo1 inhibitors for neuropsychiatric and anti-epileptic drug development. *Biochem. Soc. Trans.* 2014; 42:461–7. [PubMed: 24646261]
- [17]. Distler MG, Palmer A. a. Role of Glyoxalase 1 (Glo1) and methylglyoxal (MG) in behavior: recent advances and mechanistic insights. *Front. Genet.* 2012; 3:250. [PubMed: 23181072]
- [18]. Ahmed N, Thornalley PJ. Advanced glycation endproducts: what is their relevance to diabetic complications? *Diabetes. Obes. Metab.* 2007; 9:233–45. [PubMed: 17391149]
- [19]. Brownlee M. Biochemistry and molecular cell biology of diabetic complications. *Nature.* 2001; 414:813–20. [PubMed: 11742414]

Highlights

- Mice overexpressing *Glo1* only in neurons showed increased anxiety-like behavior
- Microinjection of MG, the substrate for *Glo1*, into BLA reduced anxiety-like behavior
- *Glo1* and MG regulate anxiety-like behavior via well-established central mechanisms

Fig.1. Neuron specific overexpression of *Glo1* increases anxiety-like behavior in the open field test (OFT)

A) Construct map illustrating the generation of FLOXGlo1KI mice wherein *Glo1* with a preceding floxed STOP was knocked-in to ROSA26 locus. B) Western blot indicating non-significant ($p=0.054$) increases in GLO1 in the brain of FLOXGLO1KI $\times$ SynCRE mice relative to their FLOXGLO1KI $\times$ WT littermates. Inset: representative western blots of GLO1 and α -tubulin from FLOXGLO1KI x WT (WT) and FLOXGLO1KI x Syn-CRE (CRE) mice. C) Increased GLO1 enzymatic activity was seen in the brain, (D) but not liver of FLOXGLO1KI $\times$ Syn-CRE mice relative to their FLOXGLO1KI $\times$ WT littermates. E) Mice

overexpressing *Glo1* in neurons (FLOXGlo1KI $\times$ Syn-CRE) show reduced center duration in the OFT, indicating increased anxiety-like behavior. F) Mice did not differ in total distance traveled. Data are mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$

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Fig.2. Bilateral microinjection of methylglyoxal into the basolateral-amygdala (BLA) reduces anxiety-like behavior in the OFT

A) Schematic representation of bilateral microinjections sites. Images were adapted from Paxinos and Franklin (2004), the color of the circles is defined below the figure and corresponds to column color in panels B and C indicating the nature of the injection. B) Bilateral microinjection of MG (12 μ M or 24 μ M) or Midazolam (4mM) directly into the BLA increased center duration in the OFT over 30min compared to vehicle (VEH) treatment, indicating reduced anxiety-like behavior. C) There was no effect of these treatments on total distance traveled. Data are mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$ by Holm-Sidak Post-hoc comparisons to vehicle.