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RNA Interference Suggests a Primary Role for Monoacylglycerol Lipase in the Degradation of the Endocannabinoid 2-Arachidonoylglycerol

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Running Title: RNAi-mediated silencing of MGL enhances 2-AG accumulation

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ABSTRACT

The endogenous cannabinoid, 2-arachidonoylglycerol (2-AG), is produced by neurons and other cells in a stimulus-dependent manner and undergoes rapid biological inactivation through transport into cells and catalytic hydrolysis. The enzymatic pathways responsible for 2-AG degradation are only partially understood. We have previously shown that overexpression of monoacylglycerol lipase (MGL), a cytosolic serine hydrolase that cleaves 1- and 2-monoacylglycerols to fatty acid and glycerol reduces stimulus-dependent 2-AG accumulation in primary cultures of rat brain neurons. We report now that RNA interference (RNAi)-mediated silencing of MGL expression greatly enhances 2-AG accumulation in HeLa cells. Following stimulation with the calcium ionophore, ionomycin, 2-AG levels in MGL-silenced cells were comparable to those found in cells in which 2-AG degradation had been blocked using methyl arachidonyl fluorophosphonate (MAFP), a non-selective inhibitor of 2-AG hydrolysis. The results indicate that MGL plays an important role in the degradation of endogenous 2-AG in intact HeLa cells. Furthermore, immunodepletion experiments show that MGL accounts for at least 50% of the total 2-AG-hydrolyzing activity in soluble fractions of rat brain, suggesting that this enzyme also contributes to 2-AG deactivation in the central nervous system.

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INTRODUCTION

The endocannabinoids are a class of lipid-derived neuromodulators that act as local messengers in the brain, influencing neuronal activity and neurotransmitter release (Brager et al., 2003; Freund et al., 2003; Piomelli, 2003). The biological actions of these compounds, which include anandamide and 2-arachidonoylglycerol (2-AG), are mediated through G protein-coupled cannabinoid receptors and are terminated by high-affinity transport into cells followed by enzymatic hydrolysis (Beltramo et al., 1997; Beltramo and Piomelli, 2000; Hillard et al., 1997). Biochemical, pharmacological, and genetic evidence suggest that anandamide hydrolysis in the brain is primarily catalyzed by fatty acid amide hydrolase (FAAH) (Cravatt and Lichtman, 2003). Pharmacological blockade of FAAH activity or deletion of the *faah* gene markedly decreases anandamide degradation in the rodent central nervous system (Cravatt et al., 2001; Kathuria et al., 2003). Although early studies have shown that FAAH can hydrolyze both anandamide and 2-AG at comparable rates *in vitro* (Goparaju et al., 1998; Lang et al., 1999; Patricelli and Cravatt, 1999), more recent evidence suggests that the role of this enzyme in terminating 2-AG signaling *in vivo* may be limited. For example, administration of the selective FAAH inhibitor URB597, which greatly reduces anandamide degradation in the rat brain, has no effect on 2-AG levels (Kathuria et al., 2003). Moreover, 2-AG breakdown is preserved in mutant *faah*$^{-/-}$ mice, in which anandamide hydrolysis is almost completely absent (Lichtman et al., 2002). These findings suggest that the intracellular breakdown of anandamide and 2-AG may proceed through distinct enzymatic pathways.

In a previous study, we molecularly cloned rat brain monoacylglycerol lipase (MGL), a cytosolic serine hydrolase that cleaves 1- and 2-monoacylglycerols into fatty

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acid and glycerol (Karlsson et al., 1997), and examined its role in neuronal 2-AG deactivation. We found that MGL is abundantly expressed in discrete areas of the rat brain, including the hippocampus, cortex, and cerebellum — where CB_1 receptors are also found. We have further shown that adenovirus-induced overexpression of MGL in primary cultures of rat brain neurons curtails the accumulation of 2-AG elicited by activation of glutamate N-methyl-(D)-aspartate receptors (Dinh et al., 2002). Although these experiments indicate that MGL overexpression enhances 2-AG hydrolysis in intact neurons, they do not directly examine whether this enzyme is involved in the physiological breakdown of 2-AG. To investigate further this question, in the present study we have taken two complementary approaches. First, we have silenced MGL expression in HeLa cells using RNA interference (RNAi) (Fire et al., 1998) and examined the impact of MGL knockdown on endogenous 2-AG degradation. Second, we have used immunodepletion to determine the quantitative contribution of MGL to the total 2-AG-hydrolyzing activity present in rat brain cytosol.

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MATERIALS AND METHODS

Chemicals. Methyl arachidonyl fluorophosphonate (MAFP) was purchased from Cayman Chemicals (Ann Arbor, MI) and ionomycin from Sigma Aldrich (St. Louis, MO). The drugs were dissolved in dimethyl sulfoxide (DMSO) before use (final concentration, 0.1%).

Adenovirus Production and Cell Infections. We produced adenovirus as described (Dinh et al., 2002). Briefly, we subcloned rat MGL cDNA into the plasmid pACYC (pACYC-MGL), cotransfected pACYC-MGL or pACYC (5 μg each) with pJM17 into low-passage human embryonic kidney (HEK) 293 cells by calcium phosphate precipitation, and isolated adenovirus particles. The adenovirus stock was amplified and titered at the Viral Vector Center of the University of California, Irvine. 48 h before experiments, we infected HeLa cells for 2 h at 37°C with Ad5-Pac (control) or Ad5-MGL at a multiplicity of infection of 50.

Lipid Analyses. We extracted lipids with chloroform: methanol (2:1, vol:vol) and analyzed lipid products by high-performance liquid chromatography/mass spectrometry (HPLC/MS) as described (Giuffrida et al., 2000). 2-[$^{2}H_{8}$]AG was purchased from Cayman Chemicals. A separate standard curve was created to measure the levels of 2-oleoylglycerol using 1,3-heptadecanoyl-glycerol (500 pmol) (NuCheck Prep, Elysian, MN) as a standard.

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RNA and Protein Analyses. We isolated total RNA (RNAqueous™, Ambion, Austin, TX) from the brains of Wistar rats (250–300 g) and HeLa cells. We homogenized brains in Tris buffer (50 mM, pH 8.0) containing 0.32 M sucrose and prepared supernatant fraction by ultracentrifugation (100,000 X *g*). Protein concentration was determined with Coomassie Plus kit (Pierce Endogen, Rockford, IL) by using bovine serum albumin as a standard. We electrophoresed supernatant proteins (50 μg) on SDS-12% polyacrylamide gel, transferred them to Immobilon™ membranes (Millipore, Billerica, MA), and conducted Western blot analyses as described (Dinh et al., 2002) by using an ECL-Plus kit (Amersham Biosciences, Piscataway, NJ) to visualize immunoreactive bands.

Enzyme Assays. We incubated supernatant protein (50 μg) in Tris buffer (50 mM, pH 8.0) with 2-oleoyl-[^{3}H]glycerol (10 μM; 10,000 cpm, American Radiolabeled Chemicals, St. Louis, MO) for 30 min at 37°C. The reactions were stopped, and products were separated by organic solvent extraction (chloroform: methanol, 1:1). We measured [^{3}H]glycerol released in the aqueous phase by liquid scintillation counting.

RNA Interference. Two complementary 64 base pair oligonucleotides containing both sense and antisense 21-mer sequences from the human MGL cDNA and separated by a short hairpin sequence were synthesized; forward: 5'-GATCCCAGTGCTCAACCTTGTGCTGTTCAAGAGACAGCACAAGGTTGAGCAC TTTTTTTGG AA-3'; reverse: 5'-AGCTTTTCCAAAAAAAGTGCTCAACCT TGTGCTGTCTCTTGAACAGCACAAGGTTGAGCACTGG-3', Integrated DNA Technologies, Coralville, IA). Forward and reverse oligonucleotides were incubated in

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annealing buffer (100 mM potassium acetate, 30 mM HEPES-KOH (pH 7.4) and 2 mM magnesium acetate) for 3 min at 90°C followed by incubation for 1 h at 37°C. The annealed DNA was ligated to linearized pSilencer 2.1-U6 siRNA expression vector (Ambion) at *Bam*HI and *Hind*III sites to generate pSIL-siMGL. A sequence not found in the human, mouse, or rat genome was used as a negative control (pSIL-NC) (Ambion). For stable transfection, we seeded HeLa cells at a density of 60-75% in 6-well plates and grown overnight. The following day, we transfected cells with pSIL-siMGL or pSIL-NC (5 μg each) using Trojene™ (Avanti, Alabaster, AL) according to manufacturer's instructions. 48 h later, the culture medium was replaced with medium containing 200 μg/ml hygromycin B (Calbiochem, La Jolla, CA). Stable clones were isolated after 14 days and maintained in hygromycin B (100 μg/ml).

Real-Time Quantitative Polymerase Chain Reaction. Reverse transcription of 2 μg of total RNA was carried out with 0.2 μg of Oligo(dT)$_{12–18}$ primer for 50 min at 42°C using Superscript II RNaseH reverse transcriptase (Invitrogen, Carlsbad, CA), and real-time quantitative polymerase chain reaction was done with an ABI PRISM 7700 sequence detection system (Applied Biosystems, Foster City, CA). We designed primer/probe sets with Primer Express software (Applied Biosystems) and gene sequences available from GenBank database. Primers and fluorogenic probes were synthesized by TIB Molbiol (Adelphia, NJ). The primer/probe sequences for the human MGL gene were as follows: forward, 5'-CAACCTTGTGCTGCCAAAC-3'; reverse, 5'-CGAGAGAGCACGCTGGAG-3'; taqman probe, 5'-TGTCCCTCGGGCCCATCG-3'.

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Starting RNA levels were quantified by using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as external standard.

Stimulation of 2-AG Accumulation. We seeded HeLa cells stably expressing pSIL-siMGL (HeLa-MGL*i*) or pSIL-NC (HeLa-NC) (1 X 10^6) onto Corning® 90 mm dishes and grown to 90-95% confluency. We rinsed the cells twice for 15 min with HEPES-buffered saline (in mM: NaCl, 125; KCl, 5; $CaCl_2$; HEPES, 20 (pH 7.4); glucose, 20) and incubated cells with ionomycin (2 μM) in the same buffer for an additional 15 min to stimulate formation of 2-AG . We stopped the reaction with ice-cold methanol, extracted lipids with chloroform: methanol (2:1, vol:vol) and analyzed lipid products by HPLC/MS as described above. In some experiments, we preincubated cells with MAFP (1 μM) for 10 min and then stimulated with ionomycin. We then harvested cells in ice-cold Tris-buffered saline (50 mM, pH 8.0), prepared cell supernatant and assayed for MGL activity as described above.

Immunodepletion of MGL. We coupled 0.1 mg of affinity-purified MGL antibody or rabbit IgG to a Seize® X Immunoprecipitation column (Pierce Endogen) according to manufacturer's instructions. Supernatant protein from HeLa (25-50 μg) or brain (0.1-0.15 mg) was incubated in the antibody-coupled column for 24 h at 4°C and recovered by centrifugation (4,000 X *g*).

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Data Analyses. Results are expressed as mean ± s.e.m. One-way analysis of variance (ANOVA) or Student's *t*-test were performed, when appropriate, using Prism® (GraphPad Software, San Diego).

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RESULTS

RNAi-mediated silencing of MGL expression enhances accumulation of endogenous 2-AG

To examine the functional role of MGL in 2-AG degradation, we generated a HeLa cell line (HeLa-MGL*i*) in which MGL expression was stably silenced by RNAi (Fire et al., 1998). Real-time PCR analyses revealed an approximately 85% reduction in MGL mRNA expression in HeLa-MGL*i* when compared to wild-type or control-transfected cells (HeLa-NC) (Fig. 1*a*). Consistent with this result, MGL activity in HeLa-MGL*i* cells was approximately 20% of that found in HeLa-NC (Fig. 1*b*).

Low basal levels of 2-AG were detectable by isotope dilution HPLC/MS in both HeLa-NC and HeLa-MGL*i* cells (Fig. 1*c*, open bars). However, in HeLa-MGL*i* cells such levels were significantly higher than in control HeLa-NC cells ($P < 0.05$; Student's *t*-test) (Fig. 1*c*). Similar results were obtained when measuring the non-endocannabinoid monoacylglycerol, 2-oleoylglycerol (2-OG) (Fig. 1*d*, open bars). After a 15-min incubation with the calcium ionophore, ionomycin (2 μM), 2-AG accumulation was strongly stimulated in both cell types, but much more so in HeLa-MGL*i* than HeLa-NC cells (Fig. 1*c*, closed bars). The levels of 2-OG were also elevated (Fig. 1*d*, closed bars). The results indicate that MGL contributes to the degradation of endogenous 2-AG in intact cells, and confirm the broad role of this enzyme in the hydrolysis of cellular 2-monoacylglycerols (Karlsson et al., 1997).

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2-AG hydrolysis in HeLa cells is mediated primarily by MGL

If MGL-mediated hydrolysis is a primary route of 2-AG degradation in HeLa cells, then pharmacological blockade of 2-AG hydrolysis should increase 2-AG levels in wild-type cells to values similar to those seen in cells that do not express MGL. To test this prediction, we used the potent lipid-hydrolase inhibitor, methyl arachidonyl fluorophosphonate (MAFP). At 1 μM, MAFP reduced 2-AG hydrolysis in intact HeLa-NC cells to 15% of control (data not shown). When MAFP-treated cells were incubated with ionomycin (2 μM), the 2-AG content in these cells rose to values comparable to those measured in HeLa-MGL*i* cells (Fig. 2) suggesting that MGL-mediated hydrolysis is a primary route for 2-AG catabolism in intact HeLa cells.

MGL is a major 2-AG-hydrolyzing activity in brain supernatant

To examine the contribution of MGL to brain 2-AG hydrolysis, we assessed the effect of MGL immunodepletion on total 2-AG-hydrolyzing activity in the rat brain. We tested the specificity of our affinity-purified polyclonal antibody (Dinh et al., 2002) using supernatant fractions of HeLa cells in which rat brain MGL overexpression had been induced through adenovirus-mediated gene transfer (Dinh et al., 2002). As shown in Fig. 3*a*, immunodepletion reduced MGL activity in extracts of overexpressing cells by 80%, whereas it had no effect in extracts of vector-infected cells. Moreover, the procedure removed all MGL-like immunoreactivity from the extracts, as assessed by Western blot analyses (Fig. 3*b*). These results suggest that our antibody specifically recognizes and immunoprecipitates rat MGL, but does not significantly interact with human MGL

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constitutively expressed in HeLa cells. The two proteins have 83% amino acid identity (Karlsson et al., 2001). Alternatively, the levels of human MGL in HeLa cells may be below the detection limit of our antibody.

Next, we used the same procedure to deplete MGL from soluble fractions of rat brain tissue. Immunodepletion decreased MGL activity by 50% (Fig. 3*c*) along with a complete loss of detectable MGL immunoreactivity (Fig. 3*d*). The antibody removed both the ≈35 and ≈37 kDa MGL isoforms present in brain tissue, which are thought to arise either from alternative splicing or from as-yet-unidentified post-translational modifications (Karlsson et al., 2001). To begin to characterize the residual hydrolase activity found in brain supernatants after immunodepletion, we conducted similar immunodepletion experiments in wild-type C57Bl6J and *faah*$^{-/-}$ mice. As shown in Figure 4*a*, we found no difference in MGL activity before and after immunodepletion in the two strains, suggesting that the residual activity is likely due to an as-yet-unidentified enzyme since FAAH is absent in the soluble fraction of rat brain. However, the activity was completely abrogated by boiling (data not shown) or by the non-selective inhibitor MAFP (Fig. 4*b*).

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DISCUSSION

In the present study, we used RNAi to investigate the functional role of MGL in 2-AG degradation. We found that silencing of MGL constitutively expressed in HeLa cells produces a marked elevation of both basal and Ca^{2+}-stimulated 2-AG levels in these cells. We further found that the 2-AG content in MGL-silenced cells is comparable to that measured in control cells following pharmacological blockade of endogenous 2-AG-hydrolyzing activities. We interpret these results to indicate that MGL plays a key role in the physiological degradation of 2-AG in intact HeLa cells.

Whether this conclusion can be extended to brain neurons is still unclear. Nevertheless, the immunodepletion experiments presented here suggest that MGL may account for as much as 50% of the total MGL activity present in soluble rat brain fractions. Although the residual activity measured after MGL immunodepletion was inhibited by MAFP, it could not be attributed to FAAH because it was present in brain soluble fractions from *faah*$^{-/-}$ mice. Together, these results suggest that hydrolysis via MGL is a quantitatively significant route for 2-AG catabolism in the rat brain, but also imply that additional 2-AG-hydrolyzing enzymes may exist. In agreement with this possibility, previous work has shown that the pig brain contains at least two chromatographically distinct 2-AG-hydrolyzing activities (Goparaju et al., 1999).

In conclusion, the functional and pharmacological evidence presented in this study supports a primary role for MGL in mediating 2-AG hydrolysis in intact cells. As this endocannabinoid lipid has been implicated in a diversity of brain functions, targeting MGL may offer a rational approach for pharmacological intervention in neuroprotection,

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drug addiction and feeding (Hanus et al., 2003; Kirkham et al., 2002; Panikashvili et al., 2001; Vigano et al., 2003; Yamaguchi et al., 2001).

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Figure 1. RNAi-mediated suppression of MGL expression increases 2-AG content in HeLa cells. (a) Real-time quantitative polymerase chain reaction (PCR) of MGL mRNA expression in wild-type HeLa cells (open bar), HeLa-NC cells (negative control) (closed bar), and HeLa-MGL*i* cells (shaded bar) (n = 5). Initial quantities were determined using glyceraldehyde-3-phosphate dehydrogenase as a standard. (b) MGL activity in HeLa-NC (open bar) and HeLa-MGL*i* (closed bar) (n = 3). 2-AG (c) and 2-OG (d) content in HeLa-NC and HeLa-MGL*i* cells incubated with vehicle (open bars) or ionomycin (2 μM; 15 min) (closed bars). Results are representative of two independent experiments performed in triplicate. * $p < 0.05$; ** $p < 0.01$ *** $p < 0.001$; one-way ANOVA (1a, 1c, 1d); Student's *t*-test (1b) (n=6).

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Figure 2. Effect of MAFP on 2-AG content in HeLa cells. HeLa-NC cells were incubated with vehicle (open bars) or MAFP (1 μM) (closed bar) for 10 min and then stimulated with ionomycin (iono, 2 μM) for 15 min. HeLa-MGL*i* cells were treated with ionomycin (shaded bar) as described in Fig. 1 legend. No significant difference in 2-AG content is observed under these conditions. $p > 0.05$; Student's *t*-test (n=6). Results are representative of two independent experiments performed in triplicate.

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Figure 3. Effects of MGL immunodepletion in rat brain soluble fraction. (a) MGL enzyme activity in vector- (left panel) and MGL-infected (right panel) HeLa cells before and after MGL immunodepletion. (b) Western blot analysis of MGL-overexpressing HeLa supernatant before (UT) and after immunodepletion (ID). No MGL-like immunoreactivity was detected in supernatant from vector-infected (Vec) or MGL-immunodepleted HeLa cells. (c) MGL enzyme activity in rat brain soluble fraction before (open bar) and after (closed bar) MGL immunodepletion. (d) MGL-like immunoreactivity is absent in MGL-immunodepleted (ID) but not rabbit IgG-treated soluble fractions. ** $p < 0.01$ and *** $p < 0.001$; Student's *t*-test (n = 6).

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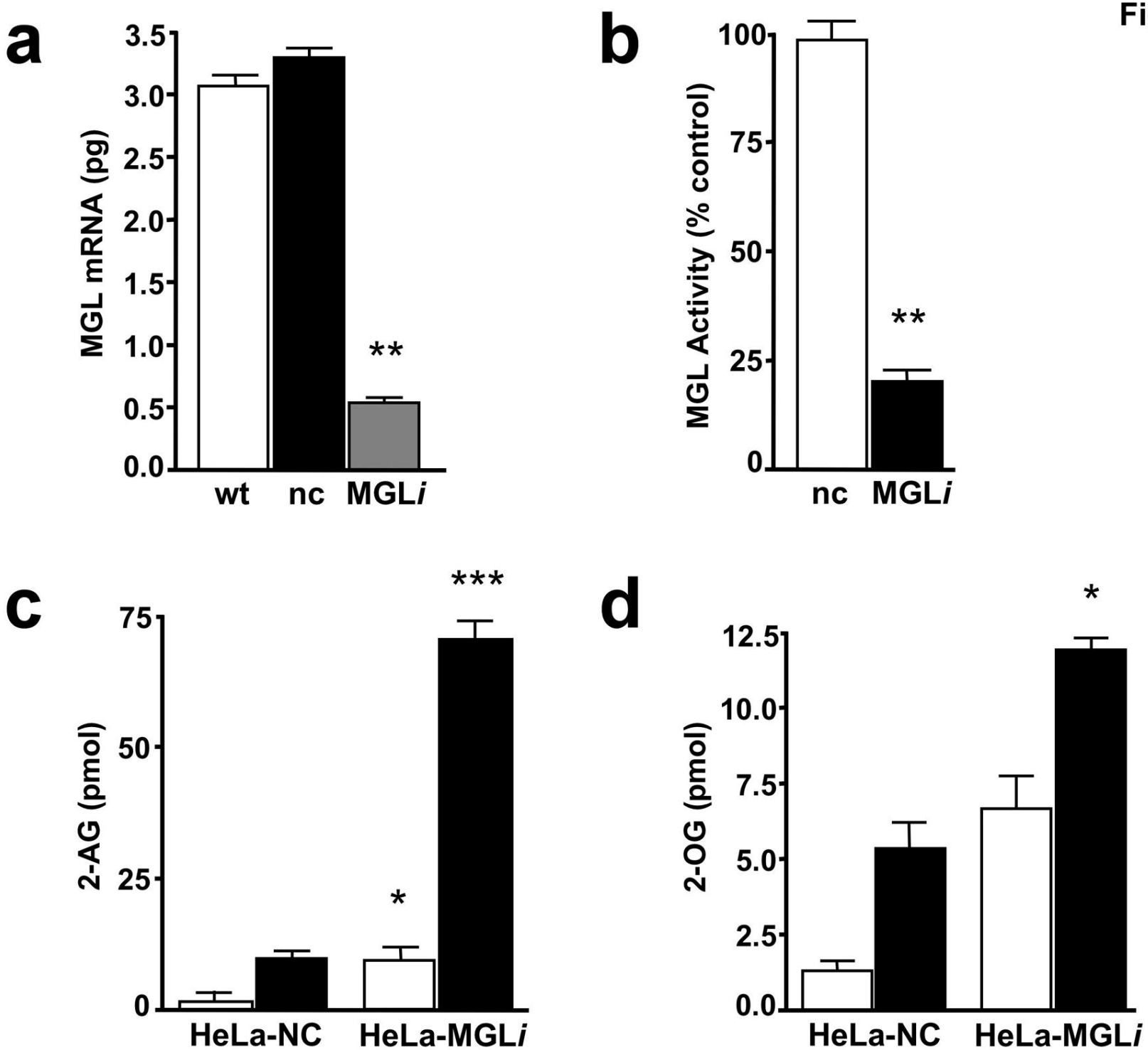
Figure 4. FAAH does not contribute to the 2-AG-hydrolyzing activity in rat brain supernatant. (a) 2-AG-hydrolyzing activity in rabbit IgG-treated (open bar) and MGL antibody-treated (closed bar) brain supernatants of wild-type and *faah*$^{-/-}$ mice (n = 3). (b) Residual 2-AG-hydrolyzing activity in MGL-immunodepleted (ID) fractions is inhibited by MAFP (n = 3).

Figure. 1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MGL mRNA (pg)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
**
wt
nc
MGLi
b
MGL Activity (% control)
100
75
50
25
0
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nc
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2-AG (pmol)
75
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2-OG (pmol)
12.5
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HeLa-NC
HeLa-MGLi

Figure 2.

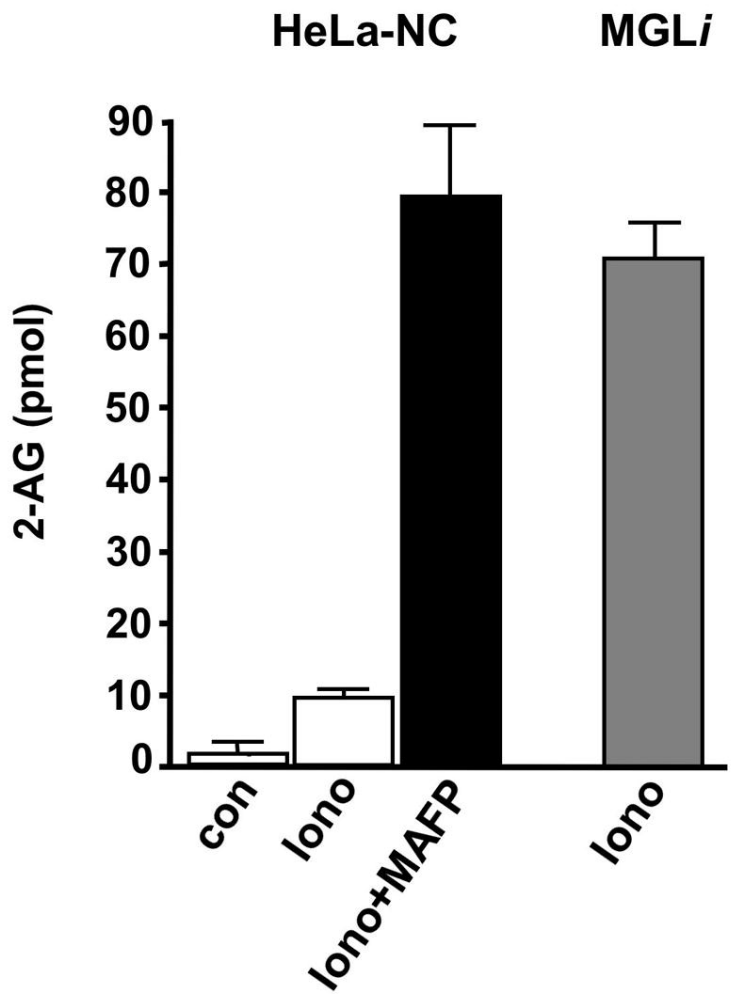

HeLa-NC
MGL*i*
2-AG (pmol)
90
80
70
60
50
40
30
20
10
0
con
Iono
Iono+MAFP
Iono

Figure 3

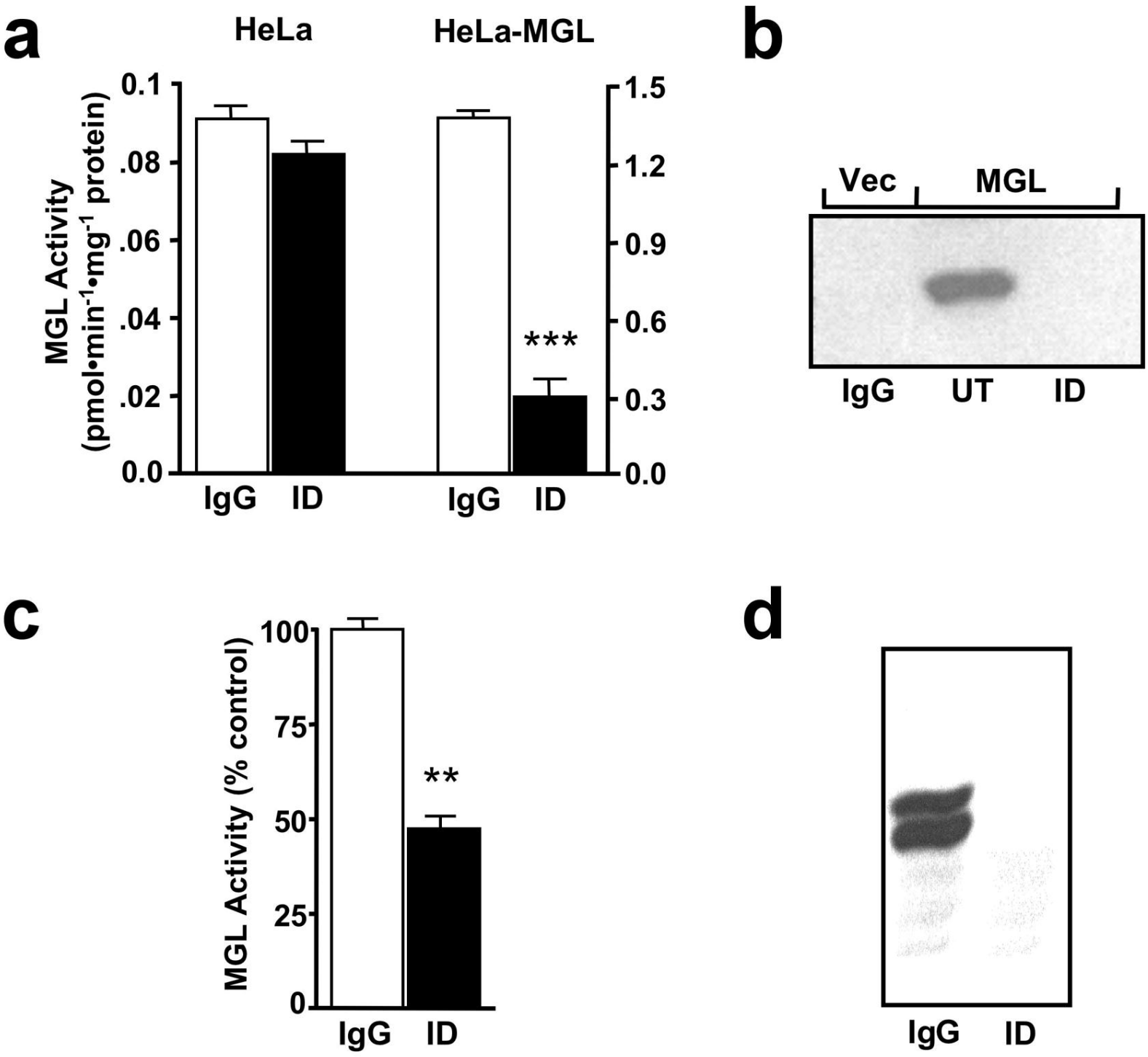
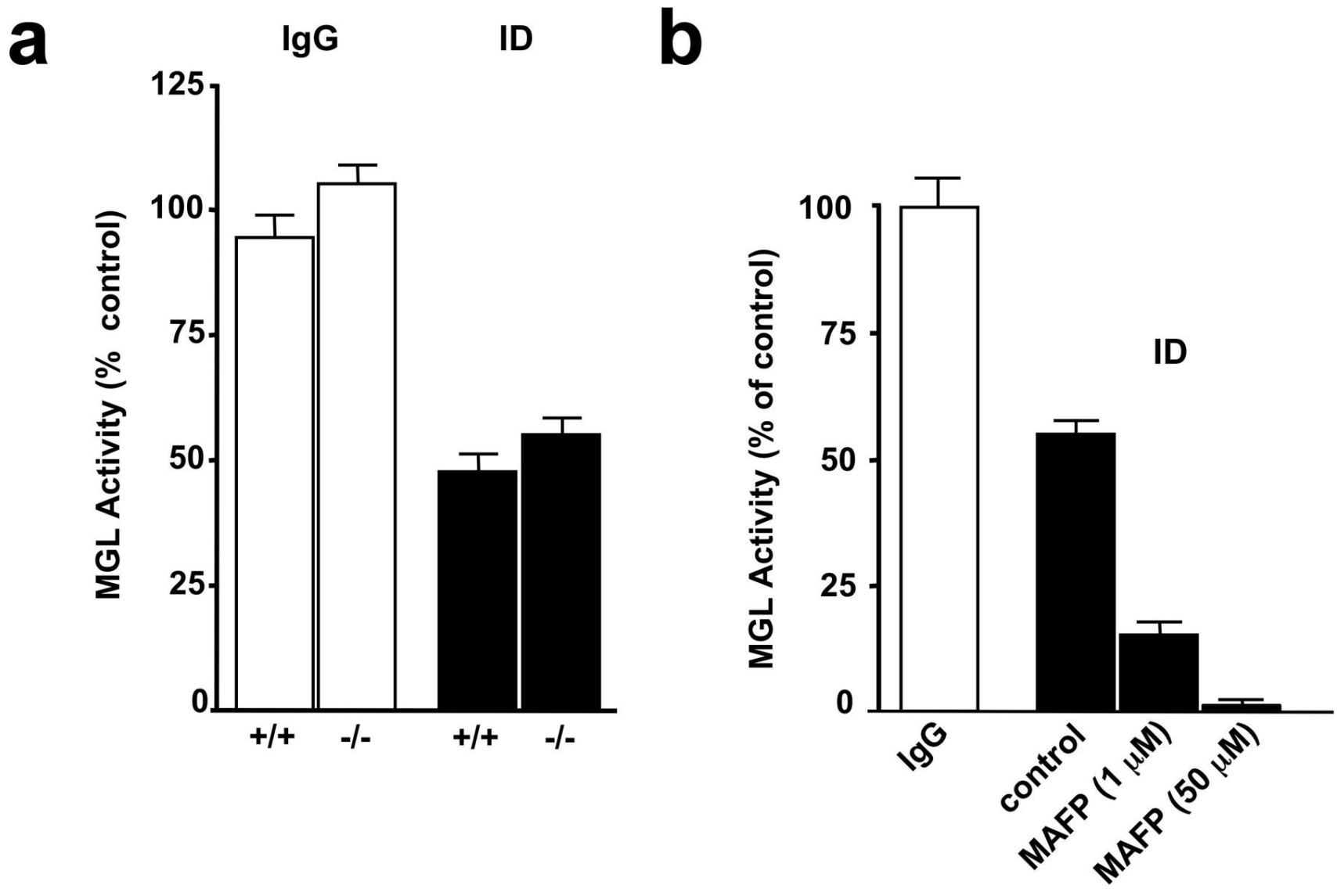

a
HeLa
HeLa-MGL
MGL Activity (pmol•min-1•mg-1 protein)
0.1
.08
.06
.04
.02
0.0
1.5
1.2
0.9
0.6
0.3
0.0
***
IgG
ID
IgG
ID
b
Vec
MGL
IgG
UT
ID
c
MGL Activity (% control)
100
75
50
25
0
**
IgG
ID
d
IgG
ID

Figure 4.


a
IgG
ID
MGL Activity (% control)
125
100
75
50
25
0
+/+
-/-
+/+
-/-
b
MGL Activity (% of control)
100
75
50
25
0
ID
IgG
control
MAFP (1 μM)
MAFP (50 μM)