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Curcumin, quercetin, and tBHQ modulate glutathione levels in astrocytes and neurons: importance of the glutamate cysteine ligase modifier subunit

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Abstract

A decrease in GSH levels, the main redox regulator, can be observed in neurodegenerative diseases as well as in schizophrenia. In search for substances able to increase GSH, we evaluated the ability of curcumin (polyphenol), quercetin (flavonoid), and *tert*-butylhydroquinone (tBHQ) to up-regulate GSH-synthesizing enzymes. The gene expression, activity, and product levels of these enzymes were measured in cultured neurons and astrocytes. In astrocytes, all substances increased GSH levels and the activity of the rate-limiting synthesizing enzyme, glutamate cysteine ligase (GCL). In neurons, curcumin and to a lesser extent tBHQ increased GCL activity and GSH levels, while quercetin decreased GSH and led to cell death. In the two cell types, the gene that showed the greatest increase in its expression was the one coding for the modifier subunit of GCL (GCLM). The increase in mRNA levels of GCLM was 3 to 7-fold higher than that of the catalytic subunit. In astrocytes from GCLM-knock-out mice showing low GSH (−80%) and low GCL activity (−50%), none of the substances succeeded in increasing GSH synthesis. Our results indicate that GCLM is essential for the up-regulation of GCL activity induced by curcumin, quercetin and tBHQ.

Keywords: curcumin, glutamate cysteine ligase, glutathione, quercetin, schizophrenia, *tert*-butylhydroquinone.

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GSH is one of the major cellular antioxidant and redox regulator, and therefore protects cells against oxidative stress (for reviews see Meister and Anderson 1983; Lu 2008). An intact GSH system is primordial to neutralize reactive oxygen species (ROS) produced by the intense metabolism and oxygen consumption that occur in the brain. A deficit in brain GSH has been observed in neurodegenerative diseases [e.g., Alzheimer's, Parkinson's, or Huntington's disease (for a review see Dringen and Hirrlinger 2003)], and in schizophrenia (Do *et al.* 2000; Yao *et al.* 2006). In schizophrenia, several lines of evidence suggest an impairment in GSH synthesis (Tosic *et al.* 2006; Gysin *et al.* 2007) that could lead to some of the pathophysiology observed in this illness (for a review see Do *et al.* 2008). Following these observations, a clinical study was conducted, during which *N*-acetyl-cysteine, a GSH precursor, was administered to schizophrenia patients. *N*-acetyl-cysteine reduced clinical severity and negative symptoms (Berk *et al.* 2008), and improved the mismatch negativity (Lavoie *et al.* 2008), a component of the auditory evoked potentials that is impaired in schizophrenia (Catts *et al.* 1995; Javitt *et al.* 1993; Shelley *et al.* 1991; for review see Turetsky *et al.* 2007). These improvements strongly suggest that using substances for boosting the GSH synthesis could be a therapeutic tool for schizophrenia.

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Abbreviations used: γ-GC, γ-glutamylcysteine; 2-VP, 2-vinylpyridine monomer; ARE, antioxidant response element; BSO, L-buthionine-(*S,R*)-sulfoximine; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethylsulfoxide; GCL, glutamate cysteine ligase; GCLC, glutamate cysteine ligase catalytic subunit; GCLM, glutamate cysteine ligase modifier subunit; GSS, GSH synthetase; KO, knock-out; LDH, lactate dehydrogenase; NDA, 2,3-naphthalenedicarboxaldehyde; ROS, reactive oxygen species; SSA, 5-sulfosalicylic acid; tBHQ, *tert*-buthylhydroquinone; WT, wild-type.

The synthesis of GSH depends on two enzymatic steps. The ligation of glutamate to cysteine is catalyzed by the rate-limiting enzyme, the glutamate cysteine ligase (GCL), and the coupling of γ-glutamylcysteine (γ-GC) with glycine is catalyzed by the GSH synthetase (GSS). During a toxic or oxidative challenge, the net decrease in GSH levels will result in an increase in its production. This is due, in part, not only to the decrease in the feedback inhibition by GSH on GCL (Richman and Meister 1975) but also to the induction of phase II detoxification enzymes and antioxidant proteins (Ishii *et al.* 2002). The genes coding for these proteins could be up-regulated following the composition of the activator protein-1 complex or the nuclear factor-κB complex (Dickinson *et al.* 2004), but the expression of these genes is mainly regulated by the antioxidant response element (ARE). ARE is a *cis*-acting regulatory element found in promoter regions of phase II and antioxidant genes (Rushmore *et al.* 1991). ARE activation signals dissociate the cytoplasmic Nrf2-Keap1 complex, allowing the transcription factor Nrf2 to translocate into the nucleus where it binds to the ARE and transcriptionally activates downstream target genes (Jaiswal 2004). Among these genes that contain an ARE sequence in their promoter, several are GSH-related genes: Nrf2 itself (Kwak *et al.* 2002), the catalytic (GCLC; Mulcahy *et al.* 1997) and the modifier (Moinova and Mulcahy 1998) subunits of GCL, as well as the specific subunit (xCT; Sasaki *et al.* 2002), and the ubiquitous subunit (4F2; Sato *et al.* 2004) of the cystine-glutamate antiporter. This exchanger allows the transport of one glutamate outside the cell, while one cystine enters, thus providing cysteine, the limiting precursor in the synthesis of GSH. In the search for substances able to induce GCL activity and up-regulate GSH levels in brain cells with compromised GSH synthesis as in schizophrenia (Do *et al.* 2000; Yao *et al.* 2006), we investigated three chemically distinct classes of substances known to increase GSH levels in various cell types: curcumin, quercetin, and *tert*-buthylhydroquinone (tBHQ). Curcumin and quercetin have been selected for they are potentially safe and easy-to-use as potential therapeutic tool, while tBHQ was mainly chosen for its well-studied action of inducer of ARE-driven transcription (Nguyen *et al.* 2003). In addition, there are evidences that these three compounds can cross the blood brain barrier (Youdim *et al.* 2004; Shih *et al.* 2005; Yang *et al.* 2005).

Curcumin, a polyphenol extracted from the rhizome of the plant *Curcuma longa* has been shown to increase GSH in various cell lines, such as cultured rat type 1 astrocytes (DI TNC1; Scapagnini *et al.* 2002), human erythroleukemia (K562; Singhal *et al.* 1999), bronchial epithelial (HBE1; Dickinson *et al.* 2003), myelomonocytes (U937; Strasser *et al.* 2005), and alveolar epithelial cell lines (A549; Biswas *et al.* 2005). The flavonoid quercetin induced an increase in GSH levels in human breast cancer cells (MCF7; Rodgers and Grant 1998), monkey kidney derived cells (COS-1; Myhrstad *et al.* 2002), and human hepatoma cell line (HepG2; Scharf *et al.* 2003; Alia *et al.* 2005). Finally, tBHQ, a synthetic phenolic antioxidant, is known to increase GSH levels in many cell types including astrocytes, following the activation of the transcription factor Nrf2 (Lee *et al.* 2003; Vargas *et al.* 2006; Townsend *et al.* 2007). The increase in GSH levels induced by these substances in various cell types is most likely because of the induction of phase II genes. Indeed, tBHQ is a well-known inducer of these genes in many cell types including astrocytes (Eftekharpour *et al.* 2000; Shih *et al.* 2003; Kraft *et al.* 2004) and curcumin has been shown to increase Nrf2 protein levels in nuclear extracts from rat astrocytes (Scapagnini *et al.* 2006). This translocation of Nrf2 to the nucleus was accompanied by an up-regulation of some phase II enzymes in these glial cells. Finally, quercetin was shown to induce ARE-dependent genes in human breast carcinoma (Valerio *et al.* 2001) and in monkey kidney cell lines (Myhrstad *et al.* 2002). This compound can confer some protection in PC12 cells (Zhu *et al.* 2007) but to our knowledge, its effects on GSH levels have not been investigated in brain cells.

In the present study, the ability of these substances to increase GSH levels was studied in cultured astrocytes and neurons as their action on GSH synthesis and the underlying mechanisms have not been thoroughly studied in these brain cells. Since astrocytes and neurons differ by their GSH content (Dringen 2000), their transport of the precursor cysteine (Kranich *et al.* 1996), their susceptibility to oxidative stress (Ben-Yoseph *et al.* 1994; Bolanos *et al.* 1995), and their response to phase II inducers (Eftekharpour *et al.* 2000; Murphy *et al.* 2001; Kraft *et al.* 2004), we expect that the action of the three substances used would be cell-specific. Therefore, the GSH-inducing effects of the various substances and their underlying mechanisms were investigated in the two cell types at the following levels: (i) gene expression of Nrf2 of the two subunits of the cystine-glutamate exchanger (xCT and 4F2), the two subunits of GCL (GCLM and GCLC) and GSS; (ii) activity of GCL; (iii) GSH and GSSG levels; and (iv) lactate dehydrogenase (LDH) release as a marker of cell death. Finally, we also evaluated the contribution and importance of GCLM in the ability of these substances to increase GSH levels and GCL activity using astrocytes from GCLM knockout mice.

Experimental procedures

Materials

S/IOPS OF1 mice were purchased from Charles River (L'Arbresle, France) or breeded in our animal facility. C57BL/6 GCLM knockout (KO) mice were kindly provided by Timothy P. Dalton and Ying Chen (Center for Environmental Genetics, Cincinnati, OH, USA; Yang *et al.* 2002) and were breeded in our animal facility. Neurobasal and DMEM medium, B27 supplement with or without antioxidants as well as Quant-it Ribogreen RNA assay kit were

purchased from Invitrogen (Carlsbad, CA, USA). Fetal bovine serum was obtained from Oxoid AG (Basel, Switzerland). SV total RNA isolation kit was purchased from Promega (Madison, WI, USA) and Advanced Protein Assay from Cytoskeleton (Denver, CO, USA). TaqMan mix and probes were purchased from Applied Biosystem (Branchburg, NJ, USA). 5-Sulfosalicylic acid (SSA) and Tris(hydroxymethyl)aminomethane were obtained from Merck & Co. Inc. (Whitehouse Station, NJ, USA). L-buthionine-(*S,R*)-sulfoximine (BSO) was purchased from Acros Organics (Geel, Belgium). Finally, Sigma-Aldrich (St-Louis, MO, USA) provided us with all the other chemicals that were ATP, tBHQ, curcumin 65–70%, dimethylsulfoxide (DMSO), 5,5′-dithiobis(2-nitro-benzoic acid), EDTA, L-glutathione oxidized (GSSG), L-glutathione reduced (GSH), flutathione reductase from baker's yeast, L-LDH from rabbit muscle, NADPH, 2,3-naphthalenedicarboxaldehyde (NDA), poly-L-ornithine, penicillin–streptomycin quercetin dihydrate, triethanolamine, and 2-vinylpyridine monomer (2VP).

Primary cultures of cortical neurons

Cultures of cortical neurons were prepared from E16-17 OF1 mice in accordance with the authorization issued by the 'Office Vétérinaire du Canton de Vaud'. The pregnant OF1 female was decapitated and brains of embryos were removed. Cortices were isolated and pooled in Hanks' balanced salt solution (137 mM NaCl, 5.3 mM KCl, 0.45 mM KH_2PO_4, 0.34 mM Na_2HPO_4, 4.16 mM $NaHCO_3$, 5.5 mM glucose, and 1 mM sodium pyruvate, pH 7.4) containing penicillin (100 U/mL) and streptomycin (100 μg/mL). Cortical cells were mechanically dissociated with a Pasteur pipette, centrifuged, and resuspended in Neurobasal medium supplemented with 2% B27, 500 μM glutamine, and 30 μM glutamate. Neurons were plated on poly-L-ornithine-coated culture dishes at a density of 800 cells/mm^2. Cells were maintained at 37°C in a humidified 5% CO_2 atmosphere. In these conditions, it has been demonstrated that the amount of glial cells is less than 1% (Brewer 1995).

Primary cultures of cortical astrocytes

Astrocytes cultures from P1-3 OF1, C57BL/6 wild-type (WT), and C57BL/6 GCLM-KO mice were prepared as already described (Sorg and Magistretti 1991), in accordance with the authorization issued by the 'Office Vétérinaire du Canton de Vaud'. Cortices were dissected in DMEM medium supplemented with 10% fetal bovine serum and containing penicillin (100 μg/mL) and streptomycin (100 μg/mL). Cortical cells were mechanically dissociated through needles with decreasing diameters, centrifuged, and resuspended in the same supplemented DMEM medium. Astrocytes were harvested on poly-L-ornithine-coated dishes and left to grow at 37°C in a humidified 5% CO_2 atmosphere. In these conditions, the purity of the astrocytes cultures was higher than 95% (Do *et al.* 1997).

Treatments

Unless otherwise cited, four- to six-day-old neurons and 14- to 15-day-old astrocytes from OF1 mice were exposed for 24 h to 5, 10, 20, 50 or 100 μM of curcumin, quercetin or tBHQ, following a medium renewal. These concentrations were chosen based on other *in vitro* studies using these compounds (Scapagnini *et al.* 2002; Alia *et al.* 2005). Non-toxic concentrations of the three substances were also tested in KO and in WT astrocytes. The medium used for the treatment was made of Neurobasal supplemented with 500 μM glutamine and 2% B27 without antioxidant. Due to water insolubility, all substances were diluted in DMSO with a final DMSO concentration lower than 0.09%. In preliminary assays, the potential effect of DMSO itself was assessed by comparing cells maintained in medium with and without DMSO. For all the measurements, *t*-tests showed no differences between DMSO and no DMSO conditions. Therefore, the DMSO condition was considered as the control. For a given substance, treatments with the various concentrations and control were carried out on separate sister dishes obtained from the same culture.

Oxidized and reduced GSH

In order to assess the redox status in the cells, quantification of total GSH and oxidized GSH (GSSG) was performed with an assay based on the Tietze method (Tietze 1969) and adapted in our laboratory (Steullet *et al.* 2008). Briefly, cells were washed, scrapped out of the dish, and sonicated before an aliquot was reserved for subsequent analysis of protein content using the Advanced Protein Assay. Proteins in the remaining cell solution were precipitated with SSA and removed by centrifugation. The pH of the supernatant was adjusted with triethanolamine. For GSSG measurement, an aliquot of the supernatant was incubated for 45 to 60 min at 22°C with 2VP, which forms a stable complex with reduced GSH. The rate of increase in absorbance at 405 nm, which measures the reduction of 5,5′-dithiobis(2-nitro-benzoic acid) by GSH, was proportional to the total GSH or to the amount of GSSG when 2VP was added. Total GSH and GSSG content in cells were expressed in nmole GSH/mg proteins and nmole GSSG/mg proteins, respectively. The percentage of GSSG relative to reduced GSH (reduced GSH levels = total GSH levels − 2 × GSSG levels) was also calculated.

Glutamate cysteine ligase activity

Glutamate cysteine ligase activity was measured with a fluorescence-based microtiter plate (White *et al.* 2003) and adapted in our laboratory (Gysin *et al.* 2007). After sonication and centrifugation, an aliquot of the supernatant was reserved for subsequent analysis of protein content using the Advanced Protein Assay. Another aliquot was mixed with a reaction cocktail [400 mM Tris(hydroxymethyl)aminomethane, 20 mM L-glutamic acid, 2 mM EDTA, 20 mM sodium borate, 2 mM serine, and 40 mM ATP added freshly] in the presence or absence of BSO to block GCL activity (negative controls). The reaction was initiated by adding 2 mM cysteine. After incubation, the reaction was stopped by adjunction of SSA and the amount of γ-GC formed was quantified by measuring NDA fluorescence. NDA fluorescence in BSO-containing wells was subtracted from the fluorescence in wells that did not contain the GCL inhibitor. The amount of γ-GC formed was then assessed using dilution series of GSH solutions (GSH and γ-GC have similar affinity to NDA leading to similar fluorescence). GCL activity was expressed in nmole γ-GC/min × mg proteins.

Gene expression

After 18-h treatment, mRNA levels were measured in cultured neurons and astrocytes following a method already described (Tosic *et al.* 2006). cDNA corresponding to 10 ng of reverse-transcribed total RNA was amplified using TaqMan

gene expression assays: *Gclm* (Applied Biosystem, Mm00514996_m1), *Gclc* (Mm00802655_m1), *Gss* (Mm00515065_m1), *Nrf2* (Mm00477784_m1), *xCT* (Mm00442530_m1), and *4F2* (Mm00500521_m1). Mouse 18S ribosomal protein (HS99999901_s1) was used as endogenous control. Gene expression was calculated using the $\Delta\Delta C_t$ method (Livak and Schmittgen 2001). The difference between the C_t of 18S and the C_t of the gene of interest (ΔC_t) was calculated for the treated and the control (DMSO) samples. The ΔC_t of the control sample was then subtracted from the ΔC_t of the treatment sample ($\Delta\Delta C_t$) and linearity was achieved with the expression $2^{-\Delta\Delta C_t}$. The result obtained represented the amount of target gene in treated sample, normalized to an endogenous reference (18S) and relative to the control.

Assessment of cell viability

Cell viability was assessed by measuring the activity of LDH released in the culture medium by dying cells (adapted from Dringen *et al.* 1998). Briefly, the decrease in NADH in the presence of pyruvate was proportional to the LDH activity and it can be quantified by measuring the absorbance at 340 nm. The amount of LDH in the medium was then reported to the amount of proteins measured in the cells of the corresponding dish.

Statistical analyses

Differential effect of cell type on GSH and GSSG content and GCL activity was assessed with the *t*-test for independent samples. The effect of treatments versus control on GSH and GSSG content, on GCL activity, and on cell viability was assessed with the *t*-test for dependent samples. Because various concentrations were used, statistical significance was corrected for multiple comparisons using the sequential Bonferroni test with the Dunn-Šidák method (Sokal and Rohlf 1997). One-way multivariate ANOVA (MANOVA) with Concentration as dependent and Cell type as independent variable was used to test for differences in cell types. When significance was achieved, *post hoc* comparisons with least significant difference tests were performed. To assess the differential effects of treatments on cells from WT and KO mice, two-way ANOVAS were used with genotype and concentration as between-factors, followed by a pairwise comparisons with least square means correction. For statistical analysis of gene expression, the $\Delta\Delta C_t$ calculation (see Experimental procedures) was calculated differently (Yuan *et al.* 2006). For each gene, the ΔC_t obtained for the control and for the treated samples were subtracted from the ΔC_t obtained for a pool of cDNA reverse transcribed from RNA extracted from mice cortices. Linearity was achieved with the expression $2^{-\Delta\Delta C_t}$. The result obtained represents the amount of target gene in control or treated sample, normalized to an endogenous reference (18S) and to a stable pool. Gene expression following a treatment was then compared with gene expression in control samples with a Wilcoxon's matched pair test. For all statistical tests, significant probability level was set to $p \leq 0.05$.

Results

Effects of curcumin, quercetin, and tBHQ treatments were first assessed in astrocytes and neurons from OF1 mice, and then also in astrocytes from GCLM-KO mice and their corresponding C57BL/6 WT mice. All measurements (i.e., GSH, ratio GSSG/GSH, GCL, and LDH activity) were performed after 24-h treatments except for mRNA levels that were measured after 18-h treatments. Apart from Table 1, results are presented as the percent change relative to control.

Table 1 Total GSH levels, GCL activity, and percentage of GSSG relative to reduced GSH measured in neurons and astrocytes from OF1 mice and in astrocytes from C57BL/6 WT and GCLM-KO mice under control condition (i.e., DMSO 0.09% added to the medium for 24 h)

	Total GSH levels nmol GSH/mg protein	GCL activity nmol γ-GC/(min × mg protein)	GSSG relative to reduced GSH %
Neurons	10.63 ± 0.68	0.06 ± 0.01	2.12 ± 0.42
OF1	*n* = 31	*n* = 11	*n* = 11
Astrocytes	28.58 ± 1.31*	0.91 ± 0.07*	2.01 ± 0.21
OF1	*n* = 37	*n* = 29	*n* = 15
Astrocytes	23.98 ± 0.96**	0.63 ± 0.09	–
WT	*n* = 19	*n* = 14	
Astrocytes	4.65 ± 0.31***	0.31 ± 0.05	–
KO	*n* = 17	*n* = 17	

WT, wild-type; KO, knock-out; GCLM, glutamate cysteine ligase modifier subunit; GCL, glutamate cysteine ligase; DMSO, dimethylsulfoxide; γ-GC, γ-glutamylcysteine.
Data are means ± SEM.
*$p < 0.001$, statistically significant when compared with neurons OF1.
**$p < 0.01$, statistically significant when compared with astrocytes OF1.
***$p < 0.001$, statistically significant when compared with astrocytes WT.

Raw data for GSH, ratio GSSG/GSH, and GCL activity in astrocytes and neurons after control situation are shown on Table 1. Raw data for GSH and GCL activity in astrocytes from WT and KO mice after control situation are also presented in this table. Under control conditions, astrocytes contained 2.7 times more GSH and showed 14.4 times higher GCL activity than neurons but the ratio GSSG/GSH was similar in the two cells types. GCL activity measured in astrocytes from WT mice was equivalent to the activity measured in OF1 astrocytes, while GSH levels were lower in WT. Compared with WT, KO astrocytes showed a decrease in GSH levels (−80%; $p < 0.001$) and a trend in decrease in GCL activity (−31%). Similarly, GSH levels were significantly lower in KO neurons when compared with WT (−74%; $p < 0.001$; data not shown). However, because the basal GCL activity in neurons was very low, and the amount of cells necessary for its measurement too large, GCL activity was not quantified in KO neurons. For the same reason, treatments with substances were not carried out in KO neurons, but only in KO astrocytes.

Toxic concentrations of curcumin, quercetin, and tBHQ treatments were evaluated by measuring the LDH release (Table 2). The analyses showed that 100 μM curcumin was

Table 2 Cell death as reflected by the LDH activity in the medium after 24-h treatments with various concentrations of curcumin, quercetin, and tBHQ in astrocytes and neurons from OF1 mice

Concentration (μM)	LDH activity (Units/mg proteins)					
	Astrocytes			Neurons		
	Curcumin	Quercetin	tBHQ	Curcumin	Quercetin	tBHQ
0	0.03 ± 0.01 *n* = 15	0.04 ± 0.01 *n* = 13	0.04 ± 0.01 *n* = 19	0.05 ± 0.01 *n* = 15	0.04 ± 0.01 *n* = 11	0.05 ± 0.01 *n* = 13
5	0.03 ± 0.01 *n* = 12	0.03 ± 0.00 *n* = 11	–	0.05 ± 0.01 *n* = 13	0.04 ± 0.01 *n* = 10	–
10	0.03 ± 0.01 *n* = 13	0.04 ± 0.00 *n* = 12	0.06 ± 0.01 *n* = 14	0.06 ± 0.01 *n* = 14	0.05 ± 0.01 *n* = 10	0.06 ± 0.01 *n* = 13
20	0.03 ± 0.01 *n* = 13	0.04 ± 0.01 *n* = 12	0.04 ± 0.01 *n* = 17	0.09 ± 0.03 *n* = 13	0.05 ± 0.01 *n* = 10	0.05 ± 0.01 *n* = 13
50	0.04 ± 0.01 *n* = 13	0.04 ± 0.01 *n* = 12	0.05 ± 0.01 *n* = 17	0.07 ± 0.02 *n* = 14	0.08 ± 0.02* *n* = 11	0.12 ± 0.02* *n* = 12
100	0.12 ± 0.02* *n* = 12	0.05 ± 0.01 *n* = 13	0.35 ± 0.11* *n* = 16	0.06 ± 0.01 *n* = 12	0.08 ± 0.02* *n* = 11	0.26 ± 0.05* *n* = 10

LDH, lactate dehydrogenase; tBHQ, *tert*-buthylhydroquinone.
*$p < 0.05$, statistically significant when compared with control.
Results are means ± SEM and expressed in U/mg proteins.

toxic for astrocytes but not for neurons. In KO astrocytes, 50 μM curcumin was already lethal. In contrast to curcumin, high concentrations of quercetin (50 and 100 μM) were more toxic to neurons than to astrocytes, and 20 μM quercetin was lethal for KO astrocytes. Finally, while 100 μM tBHQ was toxic for both neurons and astrocytes, a concentration of 50 μM led to cell death only in neurons. GSH levels, GCL activity, and mRNA levels of GSH synthesis-related genes were measured in astrocytes and neurons after treatment with the various substances at non-toxic concentrations.

Curcumin

Non-toxic concentrations of curcumin induced the expression of key GSH-synthesizing genes accompanied by an increase in GSH synthesis and levels in both astrocytes and neurons (Fig. 1a and b). In astrocytes, 10 μM curcumin slightly increased the gene expression of GCLM and GSS, while 50 μM curcumin led to an increase in mRNA levels of Nrf2, xCT, 4F2, GCLC, GCLM, and GSS (Table 3). GCLM was the gene that showed the greatest expression increase. Curcumin led to an increase in GCL activity which was accompanied by a comparable, though a little lower, increase in GSH levels (Fig. 1a). For both GCL activity and GSH levels, the effect was maximum with 50 μM curcumin (+150% and +125%, respectively). No change in the content of GSSG relative to GSH was observed (data not shown). In neurons, treatment with 10 μM curcumin led to an increase in the gene expression of Nrf2, xCT, 4F2, and GCLM (Table 3). The two genes that showed the greatest increase in their expression were GCLM and xCT. Because of material limitation, GCL activity in neurons was measured only after treatment with 10 μM curcumin, concentration that was statistically the most efficient in increasing GSH levels (Fig. 1b). The increase in GCL activity (+60%) was significant and very comparable with the increase in GSH levels (+50%). No change was observed in the percentage of GSSG relative to GSH (data not shown).

Neurons and astrocytes responded differently to curcumin as confirmed by a MANOVA on GSH levels changes (percentage relative to control) with Concentration as dependent variable and Cell type as independent variable. Indeed, there was an effect of cell type with 50 μM curcumin ($p = 0.01$), meaning that, at this concentration, the increase in GSH levels observed in astrocytes was significantly larger than in neurons. In addition, neurons tended to respond to a lower concentration of curcumin than astrocytes, as 5 μM curcumin significantly increased GSH levels in neurons but not in astrocytes.

While 10 and 50 μM curcumin increased GSH levels and GCL activity in WT astrocytes, it had no effect in KO, as shown by a two-way ANOVA with Genotype and Concentration as between-factors [$F_{(2,34)} = 75.963$; $p < 0.001$ and $F_{(2,34)} = 7.231$; $p = 0.001$, respectively]. *Post hoc* pairwise comparisons showed that in WT astrocytes, 10 and 50 μM curcumin significantly increased GSH levels (40% and 150%, respectively; Fig. 2a) and GCL activity (+50% and +80%, respectively; Fig. 2b). By contrast, in KO astrocytes, neither 10 μM nor 50 μM curcumin could increase GSH levels and GCL activity

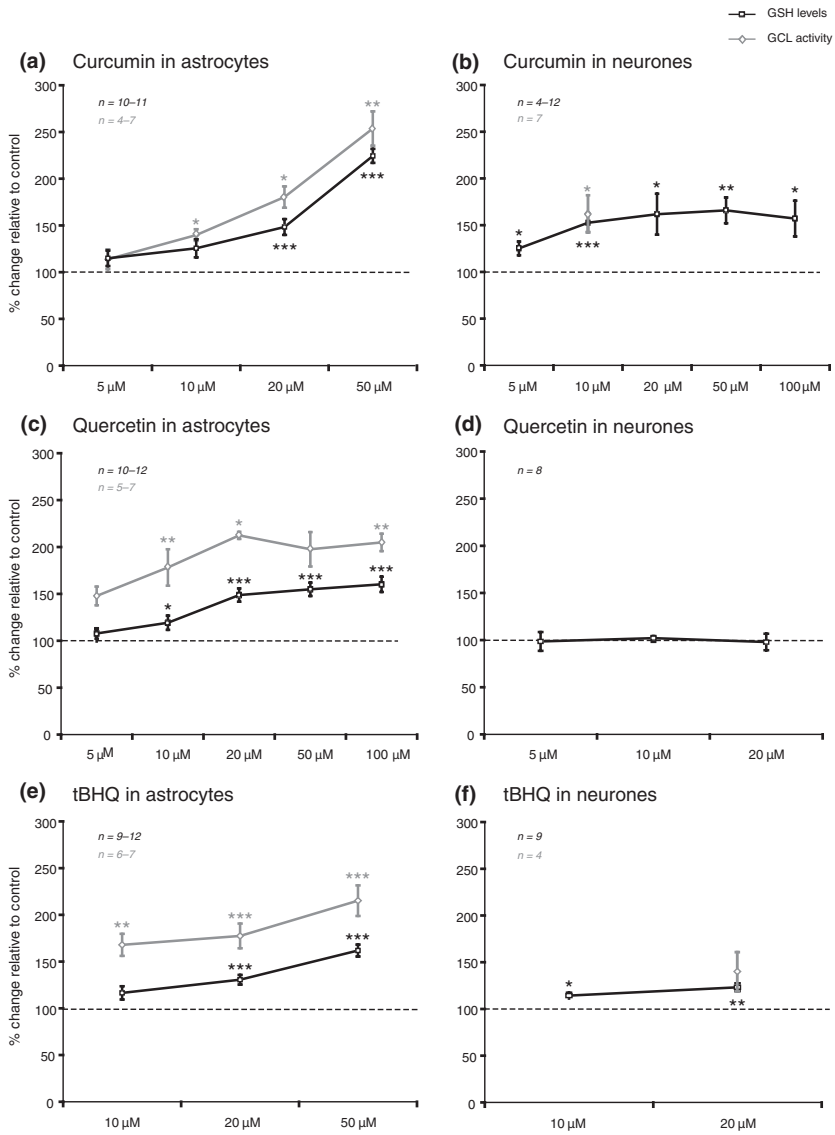

Fig. 1 Effects of curcumin, quercetin, and *tert*-buthylhydroquinone (tBHQ) on glutathione (GSH) levels and glutamate cysteine (GCL) activity in astrocytes and neurons from OF1 mice. Data are expressed as percentage of change relative to control ± SEM. Control values are indicated on Table 1. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

(Fig. 2a and b), and an increase in cell death was observed at 50 µM ($p = 0.002$; Table S1).

Quercetin

Non-toxic concentrations of quercetin induced the synthesis of GSH in astrocytes but not in neurons (Fig. 1c and d). In astrocytes, 20 µM quercetin led to an increase in mRNA levels of xCT, GCLC, GCLM, and GSS (Table 3). Like curcumin, quercetin induced the largest increase in mRNA for GCLM and xCT. The increase in GCL activity (+100%) was larger than the increase in GSH levels (+50%; Fig 1c). There was no change in the content of GSSG relative to GSH and no increase in cell death (data not shown). In neurons, non-toxic concentrations of quercetin had no effect on GSH levels (Fig. 1d). No change was observed in the percentage of GSSG relative to GSH (data not shown). As quercetin did not succeed in increasing GSH levels in neurons, the effects of this substance on GCL activity and mRNA levels were not evaluated.

Neurons and astrocytes responded differently to quercetin as confirmed by a MANOVA on GSH levels changes (percentage relative to control) with Concentration as dependent variable and Cell type as independent variable. Indeed, there was an effect of Cell type with 20 µM ($p = 0.001$), a concentration that led to an increase in GSH levels in astrocytes but not in neurons.

While 20 µM quercetin increased GSH levels and GCL activity in WT astrocytes, it had no effect in KO, as shown by a two-way ANOVA with Genotype and Concentration as between-factors [$F_{(1,10)} = 137.497$; $p < 0.001$ and $F_{(1,10)} = 10.428$; $p = 0.009$, respectively]. *Post hoc* pairwise comparisons showed that quercetin significantly increased GSH levels by about 70% in WT (Fig. 2c) and induced an even larger increase of GCL activity (+200%; Fig. 2d). By

Table 3 Effect of 18-h treatments with curcumin, quercetin, or tBHQ on the expression of GSH-related genes in astrocytes and neurons from OF1 mice

	Fold change ± SEM after treatments compared with control					
	Astrocytes				Neurons	
	Curcumin		Quercetin	tBHQ	Curcumin	tBHQ
Concentration Nb of dishes	10 μM *n* = 4	50 μM *n* = 5	20 μM *n* = 4	20 μM *n* = 7	10 μM *n* = 6	20 μM *n* = 5
Nrf2	0.97 ± 0.09	2.40 ± 0.79*	1.37 ± 0.34	1.79 ± 0.16*	1.97 ± 0.59*	3.49 ± 1.23
xCT	0.87 ± 0.21	5.46 ± 1.49*	5.47 ± 2.82	8.02 ± 3.12*	8.62 ± 2.26*	15.03 ± 5.96*
4F2	0.87 ± .015	2.66 ± 0.47*	1.41 ± 0.28	1.85 ± 0.34*	1.61 ± 0.21*	2.60 ± 0.68*
GCLC	1.04 ± 0.17	3.29 ± 0.77*	3.27 ± 0.77*	2.85 ± 0.38*	1.79 ± 0.39	3.15 ± 0.99*
GCLM	1.24 ± 0.08	15.84 ± 3.76*	14.34 ± 1.07*	5.89 ± 1.57*	6.10 ± 2.9*	9.47 ± 2.60*
GSS	1.29 ± 0.03	5.62 ± 0.42*	3.70 ± 0.58*	3.52 ± 0.67*	1.81 ± 0.55	5.1 ± 2.85
Total GSH levels	1.26 ± 0.10	2.24 ± 0.07*	1.49 ± 0.07*	1.31 ± 0.05*	1.53 ± 0.10*	1.23 ± 0.04*
GCL activity	1.40 ± 0.06*	2.54 ± 0.18*	2.12 ± 0.04*	1.77 ± 0.13*	1.62 ± 0.20*	1.40 ± 0.21

tBHQ, *tert*-buthylhydroquinone; GCL, glutamate cysteine ligase; GCLC, glutamate cysteine ligase catalytic subunit; GCLM, glutamate cysteine ligase modifier subunit; GSS, glutathione synthetase.

Values indicate the fold change in gene expression induced by each substance relative to the control condition. The effect of these substances on GSH levels and GCL activity (measured after 24-h treatments) are also presented as the fold change when compared with control.

*$p < 0.05$, statistically significant when compared with control.

contrast, quercetin did not increase GSH levels and GCL activity in KO astrocytes (Fig. 2c and d), and rather induced cell death ($p = 0.025$; Table S1).

tert-Butylhydroquinone

Non-toxic concentrations of tBHQ induced the expression of key GSH-synthesizing genes, accompanied by an increase in GSH synthesis and levels in both astrocytes and neurons (Fig. 1e and f). In astrocytes, 20 μM tBHQ increased mRNA levels of xCT, 4F2, GCLC, GCLM, and GSS (Table 3). GCLM and xCT were the genes that showed the largest increase in their expression. The increase in GCL activity (150%) was greater than the increase in GSH levels (50%). There was no change in the content of GSSG relative to GSH (data not shown). In neurons, 20 μM tBHQ led to an increase in gene expression of Nrf2, xCT, 4F2, GCLC, GCLM, and GSS (Table 3). As in astrocytes, the genes that showed the highest increase in their expression were GCLM and xCT. Because of material limitation, GCL activity was measured only after treatment with 20 μM tBHQ, the concentration that was statistically the most efficient in increasing GSH levels (Fig. 1f). A trend in an increase of about 40% in GCL activity was observed. tBHQ concentrations of 10 and 20 μM significantly increased GSH levels by about 20%. No significant change was observed in the percentage of GSSG relative to GSH (data not shown).

There was no difference in the response of neurons and astrocytes to non-toxic tBHQ concentrations as shown by MANOVA on GSH levels changes (percentage relative to control) with Concentration as dependent and Cell type as independent variable. While 20 μM tBHQ increased GSH levels and GCL activity in WT astrocytes, it had no effect on KO astrocytes as shown by a two-way ANOVA with Genotype and Concentration as between-factors [$F_{(1,27)} = 3.691$; $p = 0.06$ and $F_{(1,19)} = 5.795$; $p = 0.03$, respectively]. *Post hoc* pairwise comparisons showed that in astrocytes from WT, 20 μM tBHQ significantly increased GSH levels by about 40% ($p < 0.05$; Fig. 2e) and GCL activity by 100% ($p < 0.05$; Fig. 2f). However, 20 μM tBHQ could not increase either GSH levels or GCL activity in astrocytes from KO (Fig. 2e and f). No significant increase in cell death was observed.

Discussion

The aim of the study is to test the ability of curcumin, quercetin, and tBHQ to boost GSH synthesis in cultured astrocytes and neurons. These substances are known to increase GSH levels and/or promote protection against oxidative stress in different non-brain cells. Curcumin was studied in astrocytes and tBHQ in both glial cells and neurons but to our knowledge none of the previous studies have thoroughly analyzed and compared the effects of these substances on the synthesis of GSH in both astrocytes and neurons. Thus we have evaluated, in these two types of brain cells, the effects of curcumin, quercetin, and tBHQ on GSH levels, GCL activity, and gene expression of key proteins were implicated in the synthesis of GSH. We have also evaluated the contribution of GCLM in the induction of GSH synthesis.

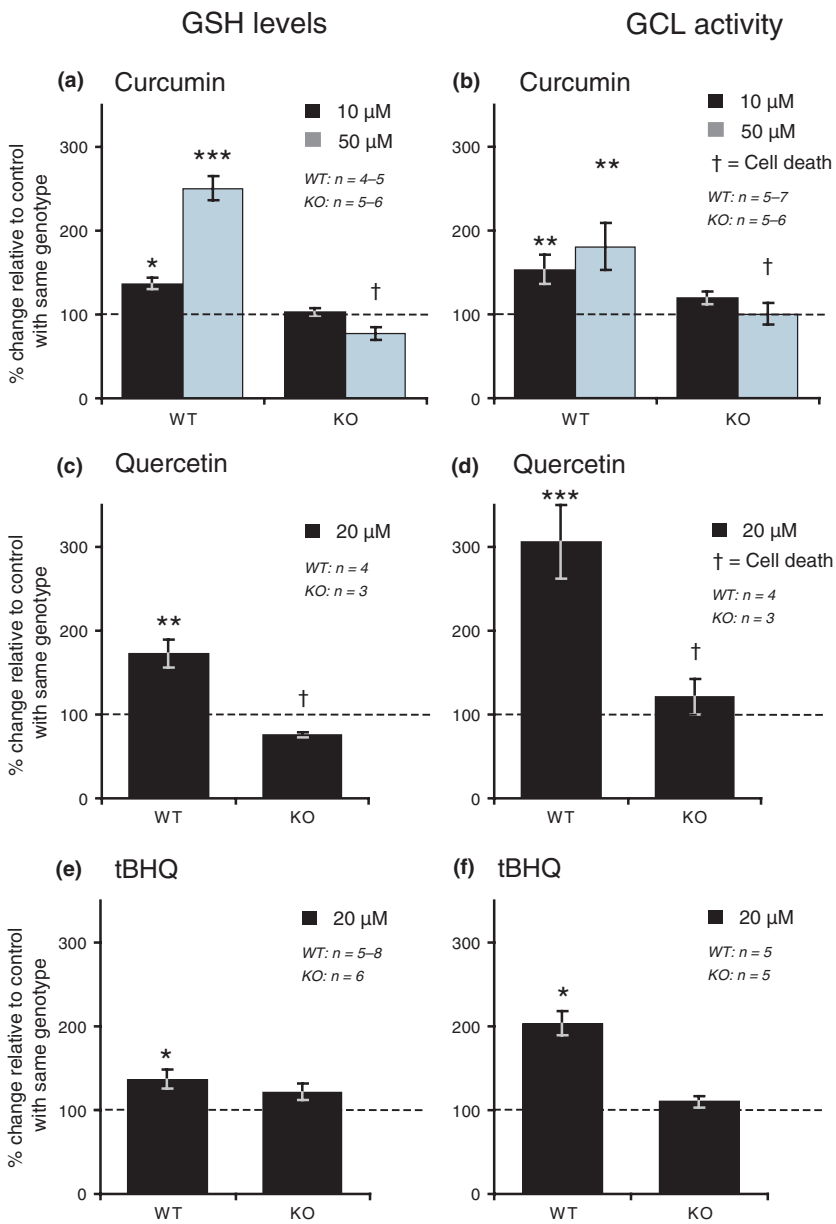

Fig. 2 Effects of curcumin, quercetin, and *tert*-buthylhydroquinone (tBHQ) on glutathione (GSH) levels, glutamate cysteine (GCL) activity, and cell death in astrocytes from C56BL/6 wild-type (WT) and glutamate cysteine ligase modifier subunit knock-out (KO) mice. Data are expressed as percentage of change relative to control ± SEM. Control values are indicated on Table 1. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ when compared with control from same genotype.

The major findings can be summarized as follows: (i) in astrocytes, all three compounds increased GSH levels and GCL activity; (ii) in neurons, only curcumin and to a lesser extent tBHQ but not quercetin increased GSH synthesis; (iii) toxic effects were different in the two cell types. High concentrations of curcumin were more toxic for astrocytes than for neurons, whereas high concentrations of quercetin and tBHQ were more toxic for neurons; (iv) All three compounds induced gene expression of most of the key proteins implicated in the GSH synthesis but GCLM and xCT were most strongly up-regulated in both astrocytes and neurons; (v) none of the substances could induce GSH synthesis in GCLM-KO astrocytes, indicating an essential role of GCLM in the up-regulation of GSH synthesis.

Differential effects of treatment in astrocytes and neurons

Neurons and astrocytes differed mostly by the extent to which the substances studied could increase GSH levels. While curcumin, quercetin, and tBHQ strongly increased GSH levels in astrocytes, only curcumin did the same in neurons. Neurons tended to respond to lower concentrations of curcumin than astrocytes but they were unable to respond as robustly as astrocytes following a strong challenge. The difference between the two cells types was not so surprising given that the expression of phase II detoxification enzymes were lower in neurons (Eftekharpour *et al.* 2000; Murphy *et al.* 2001; Kraft *et al.* 2004). Astrocytes having a more efficient GSH synthesis system and higher GSH contents than neurons can protect the latter cells against oxidative

stress and toxic compounds. Indeed, the GSH excreted from astrocytes can act as an antioxidant in the extracellular compartment and boost GSH levels in neurons by increasing the availability of cysteine (Dringen *et al.* 1999). Similarly, over-expression of xCT in glial cells led to an increased production of GSH and protected neurons against oxidative stress (Shih *et al.* 2006). Furthermore, it was proposed that Nrf2-dependent increase in glial GSH synthesis was both necessary and sufficient for the protection of neurons (Shih *et al.* 2003).

Curcumin up-regulated GSH synthesis in astrocytes and neurons

Curcumin was the only compound tested that strongly induced GSH synthesis in both astrocytes and neurons. Our results are consistent with those obtained in human lung epithelial cell lines where curcumin increased the content of *GCLC* (Biswas *et al.* 2005) and *GCLM* mRNA as well as in the content of both GCL subunit proteins (Dickinson *et al.* 2003). Furthermore, curcumin was shown to induce 1.6-fold increase in GCL activity in human erythroleukemia cells line (Singhal *et al.* 1999). Apart from its ability to increase GSH levels, curcumin shows its own protective effect. Indeed, curcumin does not have the typical ring structure of polyphenol compounds: it possesses a diketone group and two phenol rings that act as electron traps involved in metal-ligand complexation (Daniel *et al.* 2004). In rats, pre-treatment with curcumin reversed tardive dyskinesia and attenuated oxidative damage induced in the brain after chronic administration of haloperidol (Bishnoi *et al.* 2008). Unsaturated carbonyl compounds like curcumin are substrates for GSH transferases (glutathione *S*-transferase; van Iersel *et al.* 1997), therefore participating in their own elimination from the cell. However, the observed increase in GSH indicates that the formation of the conjugate curcumin-GSH is not a significant route for metabolism of curcumin as this reaction would tend to decrease GSH levels. These results were consistent with previous metabolic studies in rat liver which indicated that curcumin was metabolized primarily to glucuronides and sulfates (Holder *et al.* 1978).

Quercetin induced GSH synthesis only in astrocytes

Quercetin increased GSH synthesis in astrocytes but not in neuron cell, death being observed with high concentrations. By analogy, quercetin has been shown to be deleterious in hepatocytes (Alia *et al.* 2005), and to cause a depletion of GSH accompanied by nuclear lipid peroxidation, DNA damages, and cell death in human hepatocytes and epithelial cell lines (Sahu and Gray 1996; Duthie *et al.* 1997). Quercetin is known to exert an antioxidant effect in the presence of high levels of GSH (Ferraresi *et al.* 2005), while in an oxidizing milieu it becomes pro-oxidant. Indeed, the polyphenolic structure of quercetin scavenges free radicals and reacts with ROS to produce potentially harmful *o*-quinones (Boots *et al.* 2003). These products are then neutralized by GSH. In absence of high levels of GSH, cells might be unable to cope with the toxicity of quercetin by-products. The toxic effect of quercetin on neurons but not on astrocytes could be related to the fact that GSH levels and GCL activity are respectively about 3 and 14 times lower in neurons than in astrocytes.

In astrocytes, quercetin was less efficient than curcumin in increasing GSH levels despite a very strong up-regulation of GCL activity. This could be because of the fact that part of the newly formed GSH was immediately used to conjugate and detoxify the quercetin by-products. The lower efficacy of quercetin, and the fact that it is toxic for neurons, is consistent with a study showing that curcumin, but not quercetin, is neuroprotective in a rat model of Parkinson's disease (Zbarsky *et al.* 2005).

tert-Buthylhydroquinone increases GSH synthesis mostly in astrocytes

At non-toxic concentrations, tBHQ was highly efficient in increasing both GSH levels and GCL activity in astrocytes. However, despite the robust effect of tBHQ on gene expression in neurons, the induced increase in GCL activity and GSH levels was only marginal. These results agree with a previous study showing that tBHQ up-regulated GSH synthesis in astrocytes but not in neurons (Eftekharpour *et al.* 2000).

After quercetin treatment, the tBHQ-induced increase in GSH levels in astrocytes did not match the increase in GCL activity. tBHQ can easily be conjugated to GSH through glutathione *S*-transferase (Nakamura *et al.* 2003) and this could explain the fact that the increase in GSH levels in astrocytes is lower than the increase of GCL activity. tBHQ can also undergo oxidation-reduction reactions following a dealkylation step, and act as a pro-oxidant (Nguyen *et al.* 2003). The oxidation of tBHQ to its corresponding quinone, *tert*-butylquinone, is accompanied by the generation of ROS (Kahl *et al.* 1989). These ROS must be neutralized and, indeed, we observed a trend in an increase in the percentage of GSSG relative to GSH with 100 μM tBHQ in astrocytes. At this concentration, GSH levels were no longer significantly increased and a trend towards cell death can be observed. Similarly, in neurons, high concentrations of tBHQ led to cell death, implying that after a certain concentration, brain cells could not cope anymore with the toxic effects of tBHQ.

Induction of the cystine transporter

In both astrocytes and neurons, curcumin and tBHQ induced a strong increase in the gene expression of xCT, the specific subunit of the cystine-glutamate exchanger. This up-regulation of xCT suggests an up-regulation of the exchanger system for cystine, and consequently an increase in cystine uptake by the cells. Therefore, although neurons as opposed to astrocytes, seem to have a preference for the transport of

cysteine (Shanker *et al.* 2001) via the excitatory amino acid transporters, they also possess the cystine-glutamate exchanger (La Bella *et al.* 2007). An up-regulation of this exchanger during an oxidative challenge could provide more cysteine precursors to the neurons and boost the cellular defense mechanisms when needed.

Importance of glutamate cysteine ligase modifier subunit

For both neurons and astrocytes, all the compounds and concentrations that were efficient in increasing GCL activity and GSH levels caused particularly a strong increase in the gene expression of GCLM. Therefore, we further evaluated the importance of GCLM using astrocytes from GCLM-KO mice. In these astrocytes, the GSH levels were decreased by 80% compared to the levels observed in WT astrocytes. The activity of GCL was decreased only by 30%, in agreement with results obtained using short interfering RNA against GCLM in human kidney cells 293T (Diaz-Hernandez *et al.* 2005). Considering the abundance of GCLC relative to GCLM in WT brain (Chen *et al.* 2005), the sustained 70% of GCL activity in KO astrocytes is not surprising. This difference between the decrease in GSH levels and the reduction of GCL activity suggests that the turnover and use of GSH is very fast and that even a relatively small decrease in the capacity of synthesizing GSH because of the lack of a functional GCLM can have a drastic effect on GSH levels.

Curcumin and quercetin caused cell death in KO astrocytes, but not in WT, suggesting a greater susceptibility to oxidative stress in KO astrocytes. This is in line with the increased sensitivity to oxidative stress observed in fetal fibroblasts from GCLM-KO mice (Yang *et al.* 2002) and in GCLM-knockdown neurons (Diaz-Hernandez *et al.* 2005). We observed that curcumin, quercetin, and tBHQ failed to increase GSH synthesis in KO astrocytes. In WT astrocytes, the three substances tested up-regulated GCLC, xCT, and GSS, in addition to GCLM. If we assume that the induction of expression of these genes in astrocytes from WT and KO mice is similar, it indicates that up-regulating the cystine transport, the GCLC subunit, and GSS is not sufficient to enhance GSH synthesis in the absence of GCLM.

In WT astrocytes, the three substances induced a strong increase in GCLM mRNA levels that was accompanied by an increase in the activity of GCL, even when the increase in the gene expression of GCLC was small. This observation shows that the up-regulation of the modifier subunit is sufficient to enhance GCL activity when enough GCLC is available. The presence of GCLM is therefore necessary, but not sufficient to increase the production of GSH. Indeed, when GCLC is deficient, increasing GCLM does not improve GSH synthesis (Diaz-Hernandez *et al.* 2005). Under normal basal situation, the contribution of GCLM in the GCL activity is limited, most likely because of a high GCLC/GCLM ratio as observed in brain tissue (Chen *et al.* 2005). This high GCLC/GCLM ratio implies that there is room for up-regulation of GCLM, and therefore of GCL activity and GSH synthesis in astrocytes during an oxidative challenge. Altogether, our results suggest a key role of GCLM for the up-regulation of GSH synthesis in brain cells, and its induction should be targeted.

Conclusion

In summary, we observed that curcumin, quercetin, and tBHQ significantly increased the expression of the cystine-glutamate exchanger, the two subunits of GCL (GCLM and GCLC) and GSS in astrocytes. This was accompanied by an increase in GCL activity and GSH levels. In neurons, only curcumin and to a lesser extent, tBHQ, succeeded in modulating GSH-related genes expression in such a way that GCL activity and GSH levels were increased. The response to curcumin was different in the two types of brain cells. Neurons responded to lower concentrations of curcumin than astrocytes, but they were unable to respond as robustly as astrocytes following a strong challenge. Furthermore, neurons were more susceptible to die in presence of high concentrations of tBHQ or quercetin. Thus, our results indicate that astrocytes are more efficient than neurons in boosting their defense mechanisms after an oxidative challenge. Finally, our study clearly demonstrates the importance of GCLM for the up-regulation of GSH synthesis in brain cells. Indeed, the induction of GCLM by the substances tested was much stronger than that of GCLC, and none of the substances could lead to an increase in GCL activity and GSH levels in astrocytes from GCLM-KO mice. Therefore, the use of substances able to induce GCLM expression without toxicity in brain cells could be an interesting therapeutic approach for schizophrenia, where a deficit in GSH can be a susceptible factor or for neurodegeratives diseases where a GSH deficit is observed.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Cell death as reflected by the lactate dehydrogenase activity in the medium after 24-h treatment with 10 and 50 μM

curcumin, 20 μM quercetin and 20 μM tBHQ in astrocytes from wild-type and from GCLM knock-out mice.

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