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Adaptation to G93Asuperoxide dismutase 1 in a motor neuron cell line model of amyotrophic lateral sclerosis

The role of glutathione

Silvia Tartari[1], Giuseppina D'Alessandro[1], Elisabetta Babetto[1,*], Milena Rizzardini[1], Laura Conforti[2,*] and Lavinia Cantoni[1]

1 Department of Molecular Biochemistry and Pharmacology, Istituto di Ricerche Farmacologiche Mario Negri, Milan, Italy
2 Department of Neuroscience, Istituto di Ricerche Farmacologiche Mario Negri, Milan, Italy

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Correspondence
L. Cantoni, Laboratory of Molecular Pathology, Department of Molecular Biochemistry and Pharmacology, Istituto di Ricerche Farmacologiche Mario Negri, Via G. La Masa 19, 20156 Milan, Italy
Fax: +39 02 354 6277
Tel: +39 02 3901 4423
E-mail: cantoni@marionegri.it

***Present address**
Babraham Institute, Cambridge, UK

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Motor neuron degeneration in amyotrophic lateral sclerosis involves oxidative damage. Glutathione (GSH) is critical as an antioxidant and a redox modulator. We used a motor neuronal cell line (NSC-34) to investigate whether wild-type and familial amyotrophic lateral sclerosis-linked G93A mutant Cu,Zn superoxide dismutase (wt/G93ASOD1) modified the GSH pool and glutamate cysteine ligase (GCL), the rate-limiting enzyme for GSH synthesis. We studied the effect of various G93ASOD1 levels and exposure times. Mutant Cu,Zn superoxide dismutase induced an adaptive process involving the upregulation of GSH synthesis, even at very low expression levels. However, cells with a high level of G93ASOD1 cultured for 10 weeks showed GSH depletion and a decrease in expression of the modulatory subunit of GCL. These cells also had lower levels of GSH and GCL activity was not induced after treatment with the pro-oxidant *tert*-butylhydroquinone. Cells with a low level of G93ASOD1 maintained higher GSH levels and GCL activity, showing that the exposure time and the level of the mutant protein modulate GSH synthesis. We conclude that failure of the regulation of the GSH pathway caused by G93ASOD1 may contribute to motor neuron vulnerability and we identify this pathway as a target for therapeutic intervention.

Amyotrophic lateral sclerosis (ALS) is a fatal disease that manifests with progressive paralysis caused by the degeneration and death of large motor neurons of the spinal cord, brainstem and motor cortex. Extensive oxidative damage to neuronal tissue is found in sporadic and familial forms of ALS (SALS and FALS) [1], but the molecular mechanisms leading to these changes remain unknown.

Mutations in the gene coding for Cu,Zn superoxide dismutase (SOD1) cause 2–5% of ALS cases (FALS1) [2]. SOD1 is one of the three mammalian SOD isozymes that catalyse the dismutation of superoxide to hydrogen peroxide (H_2O_2) and water, and provide defence against oxidative stress. Extensive studies in FALS1 models showed that mutations confer new toxic properties on SOD1 rather than simply reducing the clearance of superoxide radicals [3].

One explanation proposed for this 'gain of toxic function' is that mutant SOD1 has enhanced or different oxidative activities from wild-type SOD1 (wtSOD1)

Abbreviations
ALS, amyotrophic lateral sclerosis; dox, doxycycline; EGFP, enhanced green fluorescent protein; FALS, familial amyotrophic lateral sclerosis; FALS1, mutant SOD1-linked familial amyotrophic lateral sclerosis; GCL, glutamate cysteine ligase; GCLC, catalytic subunit of GCL; GCLM, modulatory subunit of GCL; GR, glutathione reductase; GSH, glutathione; GSSG, glutathione disulfide; GST, glutathione *S*-transferase; Nrf2, nuclear factor erythroid 2-related factor 2; SALS, sporadic amyotrophic lateral sclerosis; SOD1, Cu,Zn superoxide dismutase; *t*-BHQ, *tert*-butylhydroquinone; wtSOD1, wild-type Cu,Zn superoxide dismutase.

[4]. Therefore, chronic exposure to mutant SOD1 might lead to the impairment of enzymatic or non-enzymatic antioxidant systems.

Neuronal antioxidant defences rely mainly on cellular levels of glutathione (GSH) which enable cells to function during extended periods of oxidative stress [5,6]. GSH also has a major role in maintaining the cellular thiol-disulfide redox status under reducing conditions, which is important for key cell functions [7]. In the adaptive response to oxidative stress, cells increase their GSH content by activating *de novo* synthesis [8].

GSH is synthesized by the sequential action of glutamate cysteine ligase (GCL; EC 6.3.2.2) and glutathione synthetase. GSH is a feedback inhibitor of GCL activity. GCL catalyses the rate-limiting step and produces γ-glutamylcysteine using glutamate and cysteine in an ATP-dependent reaction [9]. In higher eukaryotes, GCL is a heterodimer composed of a catalytic (GCLC) and a modulatory (GCLM) subunit encoded by evolutionarily unrelated genes on different chromosomes [10]. Regulation of GCL activity is multifaceted and can result from transcriptional, post-transcriptional and/or post-translational mechanisms [11].

Information on GSH status in ALS is very scarce. Antioxidant enzymes such as glutathione *S*-transferase (GST) show low activity in ALS [12,13], suggesting that normal handling of GSH may be altered. However, GSH binding sites in the spinal cord and GSH levels in cerebrospinal fluid were high in SALS patients [14,15], possibly because of a long-term response to chronic oxidative stress. Mice overexpressing human mutant G93ASOD1, a widely used *in vivo* ALS model [16], had low GSH levels in the lumbar spinal cord during disease progression and high glutathione disulfide (GSSG) at disease onset [17]. However, GSH and GSSG levels in transgenic mice expressing comparable amounts of human wtSOD1 protein were not studied. Wild-type and G93ASOD1 have different toxicity on motor neurons. Highly overexpressed wtSOD1 also has injurious effects, but only transgenic mice expressing mutant SOD1s develop paralysis [18].

The aim of this study was to characterize the adaptive response of the GSH pool in motor neuronal cells exposed to wtSOD1 or to its mutant form G93A, and how this response is related to modulation of the activity and/or expression of GCL. Knowledge of the strategies by which cells expressing wtSOD1 limit their damage may help improve our ability to counteract the toxicity of the mutant forms of SOD1.

We developed a conditional and a constitutive cell model for FALS1. We used the murine motor neuron-like cell line NSC-34, a well-characterized *in vitro* system for motor neuron biology and pathology, expressing wild-type and G93ASOD1. Both our conditional and constitutive model have previously been shown to reproduce aspects of the oxidative and mitochondrial toxicity of mutant SOD1 [19–21]. In this study, clones with different levels of expression of G93ASOD1 – lower or higher than murine SOD1 – were used to determine whether they differently modified the GSH pool and/or synthesis. Because FALS1 patients have only one mutant allele, clones expressing lower levels of G93ASOD1 might be a better model of motor neurons in the disease in terms of expression level. However, cells expressing a higher level of G93ASOD1 might mimic more closely the higher expression of transgenic mice, which have a high copy number of the mutant gene.

Results

Validation of the conditional FALS1 model

The SOD1 level of the conditional cell lines at their fourth passage is shown in Fig. 1B. As described in Materials and methods, wild-type and G93ASOD1 reached full expression in cells cultured without doxycycline (dox−) after dox removal between the second and third passage (Fig. 1A). Dox (1 $\mu g \cdot mL^{-1}$) permanently added to the culture medium very efficiently blocked the expression of wild-type and G93ASOD1 proteins (Fig. 1A,B). However, even in the presence of dox, a very small amount of the transfected SOD1 was expressed ($< 5\%$ of that in dox− culture by densitometric analysis). Levels of wild-type and G93ASOD1 remained fairly constant in the tTA cell lines in culture without dox (Fig. 1C) and were reproducible in cultures from different aliquots of frozen cells (data not shown). Under dox− culture conditions, human SOD1 in the lowG93A-tTA cell line was slightly lower than murine SOD1, although it was higher in the highG93A-tTA or highWT-tTA cell line (Fig. 1B,C). Differences in the expression levels of wild-type or G93ASOD1 among the various clones were confirmed in western blots performed with different amounts of cell proteins or using different exposure times for the films (data not shown).

The time course of the inhibition of expression of SOD1 and enhanced green fluorescent protein (EGFP) after addition of 1 $\mu g \cdot mL^{-1}$ of dox to fully expressing tTA cell lines was also determined. In our system, the level of SOD1 protein was greatly reduced from 24 h after addition of dox, and EGFP and SOD1 protein expression decreased in parallel, showing their coregulation (see Fig. S1 and Doc. S1).

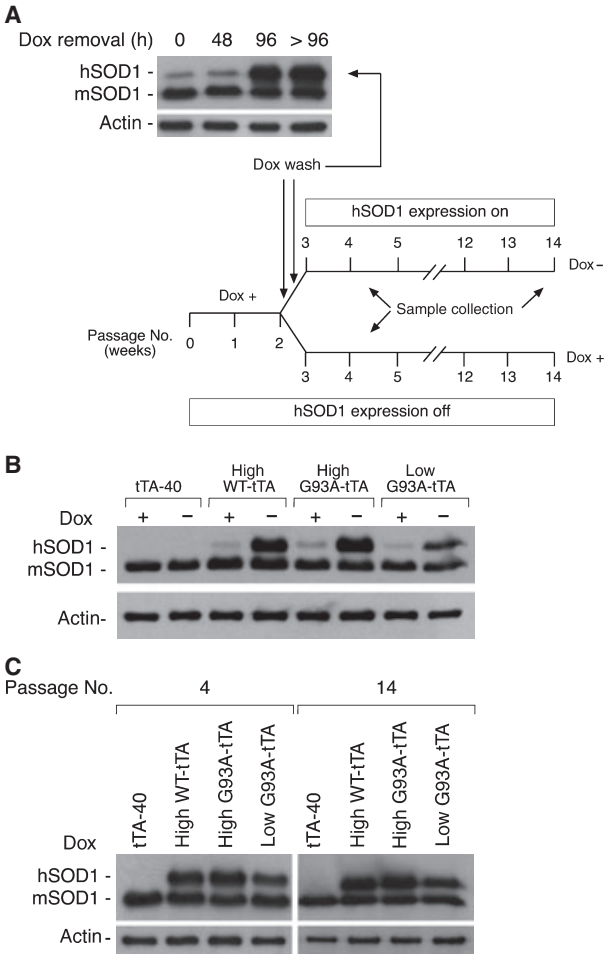

Fig. 1. Expression of wild-type or G93ASOD1 in the conditional FALS1 model. (A) Culture system and sample collection times for the conditional cell lines. Western blotting shows that removal of dox (between passages 2 and 3, as described in Materials and methods) fully induced expression of the human SOD1 (hSOD1) after 96 h (highWT-tTA cell line). (B) Expression of human wild-type or G93ASOD1 (hSOD1) evaluated by western blot of highWT-tTA, highG93A-tTA and lowG93A-tTA cell lines cultured with (+) (1 $\mu g \cdot mL^{-1}$) or without (–) dox at the fourth passage. The control tTA-40 cell line contained only murine SOD1 (mSOD1). (C) The level of wt/G93ASOD1 was constant at different passage numbers (4 and 14) in the dox– culture. Representative western blots of total cell lysates exposed together are shown.

Both wild-type and G93ASOD1 increase GSH in the conditional FALS1 model

Total GSH, GSH and GSSG were determined in the tTA-40, highWT-tTA and high/lowG93A-tTA cell lines at their fourth passage. In cells cultured without dox, this time point represents the first adaptive response to the increase in wt/G93ASOD1 expression caused by the removal of dox, whereas in cells cultured with dox, with their very low residual SOD1 expression, it represents the adaptation to constant, very low levels of wt/G93ASOD1. All the SOD1-transfected cell lines (dox–) had significantly higher total GSH than seen in tTA-40 cells and the profile of GSH content mirrored that of total GSH (Fig. 2A,B). A robust threefold increase was seen in highG93A-tTA cells. Comparable total GSH increases were also observed in dox+ cells (Fig. 2A), suggesting that this initial change takes place even with a very small extra amount of wild-type or mutant SOD1, and also that a low SOD1 expression level is somehow more effective. GSSG was also significantly increased by wild-type and G93ASOD1 overexpression, but it remained a very small percentage of GSH ($\sim 1\%$) (Fig. 2C).

GSH : GSSG ratios, $E_{hGSH/GSSG}$ values and glutathione reductase, GST activities in the conditional FALS1 model

Because the redox equilibrium of cells affects several aspects of cell homeostasis, the GSH : GSSG ratios and $E_{hGSH/GSSG}$ for cells cultured without dox were obtained (Fig. 2D,E). There was a sharp contrast in the effect of wild-type and mutant SOD1, with a significant increase in the GSH : GSSG ratio in highG93A-tTAcells. This was accompanied by a shift to a more negative value in $E_{hGSH/GSSG}$ (Fig. 2E), reinforcing the evidence of a more reduced thiol oxidation state in these cells. This did not occur in highWT-tTA cells despite the fact that both highWT- and highG93A-tTA cells had to adapt the GSH pool to overexpression of a comparably high level of human SOD1.

We next determined the specific activity of glutathione reductase (GR), essential for maintenance of the GSH : GSSG ratio. GR was no different in highWT-tTA and tTA-40 cells, but it was lower in highG93A-tTA cells than in the other cell lines (Fig. 3A). Thus increased GSSG recycling cannot explain the relative abundance of GSH over GSSG in highG93A-tTA cells. We also measured the activity of GST (Fig. 3B), a large group of proteins that use GSH to detoxify harmful products of oxidative stress. GST activity was unchanged in highWT-tTA cells, although it was lower in highG93A-tTA than in all other cell lines. This might cause lower GSH consumption in highG93A-tTA cells, therefore contributing to maintaining the high GSH levels.

In the lowG93A-tTA cell line (dox–), the GSH : GSSG ratio and $E_{hGSH/GSSG}$ did not differ from control tTA-40 or highWT-tTA cells (Fig. 2D,E).

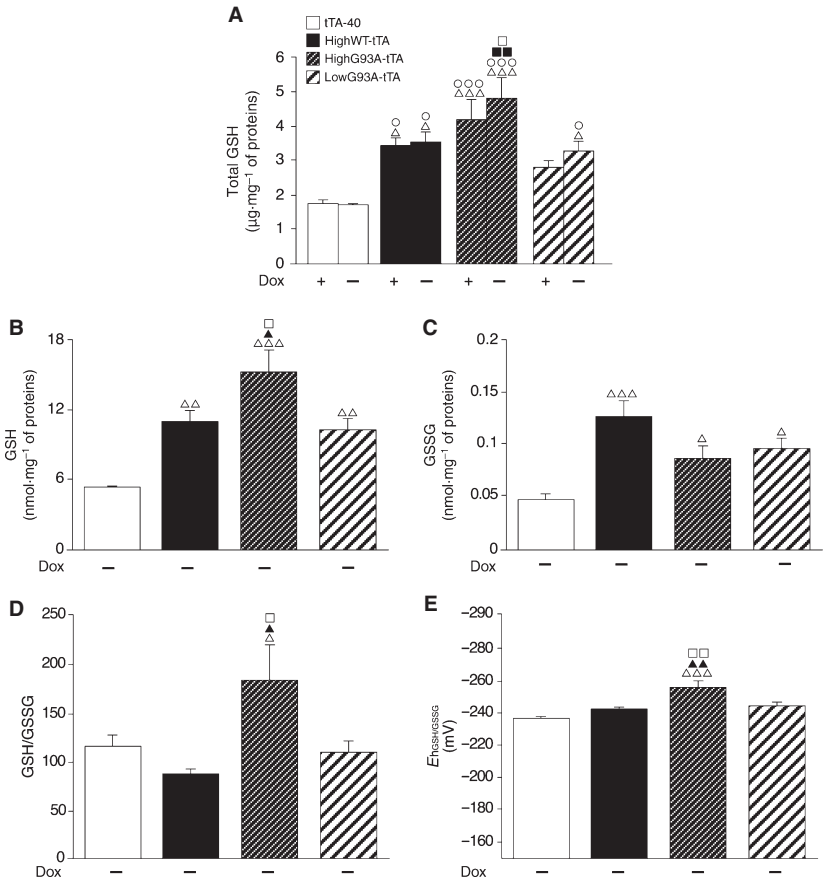

Fig. 2. GSH levels, GSH : GSSG ratio and $E_{hGSH/GSSG}$ values in the conditional FALS1 model. Levels of (A) total GSH, (B) GSH and (C) GSSG, (D) the GSH : GSSG ratio and (E) $E_{hGSH/GSSG}$ were measured in the conditional cell lines, cultured with (+) (1 μg·mL^{-1}) or without (–) dox, at the fourth passage. Values are given as mean ± SEM of four independent experiments. $\Delta P < 0.05$, $\Delta\Delta P < 0.01$, $\Delta\Delta\Delta P < 0.001$ versus tTA-40 (dox–). $\bigcirc P < 0.05$, $\bigcirc\bigcirc\bigcirc P < 0.001$ versus tTA-40 (dox +). $\blacktriangle P < 0.05$, $\blacktriangle\blacktriangle P < 0.01$ versus highWT-tTA (dox–). $\square P < 0.05$, $\square\square P < 0.01$ versus lowG93A-tTA (dox–). $\blacksquare\blacksquare P < 0.01$ versus lowG93A-tTA (dox +). (One-way ANOVA with Newman–Keuls multiple comparison post-test).

We found an increase in GR (34%, $P < 0.01$) and GST (14%) activities in comparison with tTA-40 cells (Fig. 3A,B) suggesting that when cells initially adapted themselves to overexpression of a small amount of mutant protein, they maintained the redox equilibrium changing several enzymatic activities.

Wild-type and G93ASOD1 affect the levels of GCLC and GCLM proteins differently

The increase in GSH in SOD1-transfected cell lines might result from increased synthesis. This may be because of an upregulation of the expression of GCL. We used western blotting to analyse the expression of the GCL subunits GCLC and GCLM in the dox– cultured cell lines at their fourth passage (Fig. 4). In highWT-tTA cells, GCLC remained constant, whereas GCLM showed a 34% increase over the tTA-40 value, although this change did not reach statistical significance. In lowG93A-tTA cells, both GCLM and GCLC increased significantly (95% and 90%), whereas in highG93A-tTA cells there were no significant changes, but only a small increase (15%) in GCLC. Thus, the mutant form of SOD1, more than the wild-type, modified the expression of the GCL subunits. In addition, on comparing low- and high-G93ASOD1 cells, it was evident that the induction of GCL subunits was inversely related to the expression of G93ASOD1.

In lowG93A-tTA cells (dox–), the involvement of GCL in the increase in GSH was further confirmed by measuring GCL activity, which was 16.44 ± 0.31 nmol·min^{-1}·mg^{-1} of protein, i.e. ~ 20% higher ($P < 0.01$ by Student's *t*-test) than that of tTA-40 cells (13.96 ± 0.32 nmol·min^{-1}·mg^{-1} of protein; mean ± SEM of four independent samples from two experiments).

We then treated the tTA-40 and lowG93A-tTA cell lines, both dox–, with the GCL inhibitor buthionine sulfoximine (250 μM). After 24 h, total GSH was ~ 2% of baseline (i.e. for tTA-40 and lowG93A-tTA cells, 3.35 ± 0.26 and 4.90 ± 0.30 ng·μg^{-1} protein; mean ± SE of six independent samples from two experiments, $P < 0.01$ by Student's *t*-test) indicating that, in both cell lines, GCL activity was responsible for the GSH level.

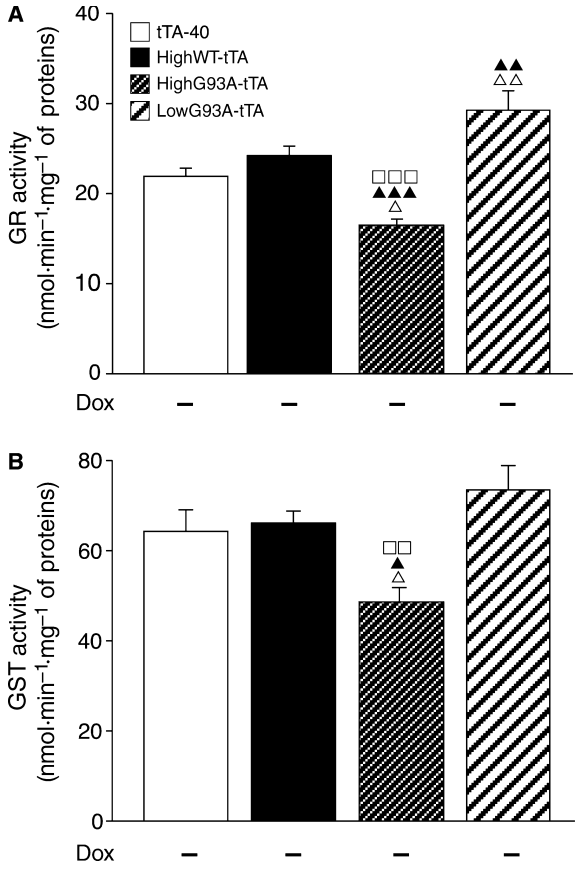

Fig. 3. GR and GST activity in the conditional FALS1 model. (A) GR and (B) GST activity were evaluated in the conditional cell lines cultured without (–) dox at the fourth passage. Values are given as mean ± SEM of three independent experiments. $\Delta P < 0.05$, $\Delta\Delta P < 0.01$ versus tTA-40. $\blacktriangle P < 0.05$, $\blacktriangle\blacktriangle P < 0.01$, $\blacktriangle\blacktriangle\blacktriangle P < 0.001$ versus highWT-tTA. $\square\square P < 0.01$, $\square\square\square P < 0.001$ versus lowG93A-tTA. (One-way ANOVA with Newman–Keuls multiple comparison post-test).

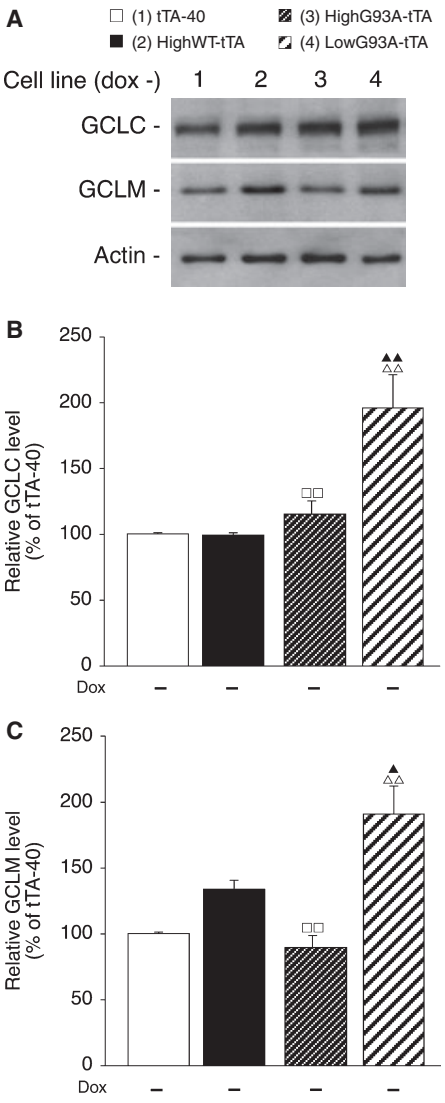

Fig. 4. Expression of GCLC and GCLM in the conditional FALS1 model. (A) GCLC and GCLM expression of the conditional cell lines cultured without (–) dox at the fourth passage. A representative western blot is shown for each protein. (B, C) GCLC and GLCM levels normalized for actin. Values are given as mean ± SEM of three independent experiments. $\Delta\Delta P < 0.01$ versus tTA-40. $\blacktriangle P < 0.05$, $\blacktriangle\blacktriangle P < 0.01$ versus highWT-tTA. $\square\square P < 0.01$ versus lowG93A-tTA (one-way ANOVA with Newman–Keuls multiple comparison post-test).

Effect of wild-type or G93ASOD1 on the GSH and protein level of GCL subunits in the constitutive FALS1 model

To confirm that the increase in GSH and expression of GCL protein subunits did not derive from some peculiarity of the conditional system, we analysed a constitutive FALS1 model, an even simpler *in vitro* system in which motor neuronal cells were never exposed to dox, did not require hygromycin B during culture and did not express EGFP. The expression levels of wild-type and G93ASOD1 in the WT-NSC and G93A-NSC cell lines resembled those of the WT/G93A-tTA cell lines cultured with dox (Fig. 5A), i.e. much lower than in the WT/G93A-tTA cell lines in dox– culture (Fig. 1B).

Total GSH was higher in both WT-NSC (57%) and G93A-NSC (66%) than in the control NSC-34 cells at their fourth passage (Fig. 5B). These increases were accompanied by significant increases in GCLC and GCLM (37% and 52%) in G93A-NSC cells only (Fig. 6A,B). Therefore, the constitutive and the conditional models responded identically, reflecting the amount and form of transfected SOD1, either wild-type or mutant.

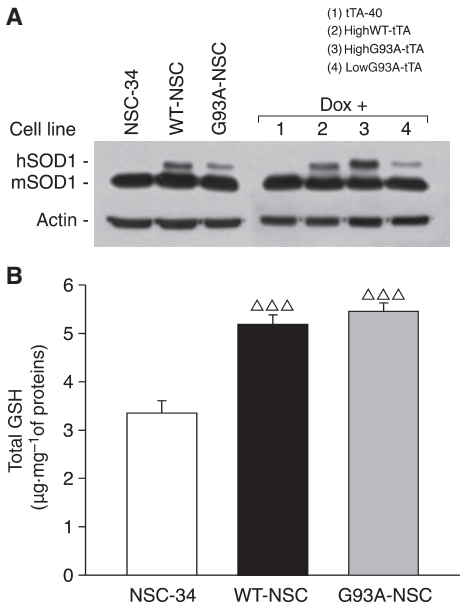

Fig. 5. Expression of wild-type or G93ASOD1 and GSH levels in the constitutive FALS1 model. (A) Expression of human wild-type or G93ASOD1 (hSOD1) in WT-NSC and G93A-NSC compared with the conditional cell lines cultured with (+) dox, determined by western blot. Thirty micrograms of protein (rather than 20 μg as in Fig. 1B for the conditional lines) were loaded for each cell line. (B) Total GSH levels of the NSC-34 and WT-NSC or G93A-NSC cell lines at the fourth passage. Values are given as mean ± SEM of four independent experiments. $\Delta\Delta\Delta P < 0.001$ versus NSC-34 (one-way ANOVA with Newman–Keuls multiple comparison post-test).

Time of exposure to wild-type or G93ASOD1 influences the GSH pool, GCL subunit protein levels and GCL activity in the conditional FALS1 model

Because FALS1 patients have long-term exposure to G93ASOD1, the effect of constant expression of wild-type and G93ASOD1 on GSH synthesis was determined at the 14th passage of the conditional cell lines in dox− culture (Fig. 7A). Total GSH in highWT-tTA cells did not differ from that in tTA-40 cells. However, it was significantly lower in highG93A-tTA cells compared with all other cell lines (∼ 30% compared with tTA-40 cells). Only lowG93A-tTA cells maintained a significant increase in the GSH pool (30% over the tTA-40 and highWT-tTA and 60% over the highG93A-tTA cells). Thus, the adaptive process of motor neuronal cells to wt/G93ASOD1 appeared to be at least biphasic, with an initial marked increase in GSH common to all the cell lines, whereas, with longer exposure, the type of SOD1 (either wild-type or G93A) and the G93ASOD1 level made the difference.

The effects of SOD1 modulation on GSH level – typical of each wild-type or G93A-tTA cell line – were reproducible in cultures from different frozen aliquots of the same clone, irrespective of the fact that over the course of the study GSH values varied slightly in the different experiments, likely reflecting subtle differences in growth and confluency of the cell cultures [22].

Levels of GCLM protein expression changed only in cells expressing the mutant protein. Thus, GCLM expression in the highG93A-tTA cell line was significantly lower than in the tTA-40, highWT-tTA and lowG93A-tTA cell lines, but was higher in lowG93A-tTA cells (20% more than tTA-40 and highWT-tTA cells), although this increase did not reach significance (Fig. 7B,C).

The activity of GCL was also measured at the same time point (Fig. 7D). In the lowG93A-tTA cell line, GCL activity was higher than in the other lines. The GCL activity in the highWT-tTA cells did not differ from the highG93A-tTA cells even though the two lines had significantly different total GSH (Fig. 7A).

Effect of *tert*-butylhydroquinone, an inducer of GSH and GCL activity, in the conditional FALS1 model

Total GSH in the highWT-tTA, highG93A-tTA and lowG93A-tTA cells (dox− cultured) was analysed 24 h after treatment with *tert*-butylhydroquinone (*t*-BHQ). All cells were at the 14th passage, the time point considered more representative of the response of cells chronically exposed to wild-type or G93ASOD1. In all the cell lines, *t*-BHQ significantly increased total GSH, but the level in highG93A-tTA cells was significantly lower than in highWT-tTA cells under basal conditions and after *t*-BHQ treatment (Fig. 8A), indicating that highG93A-tTA cells had a lower antioxidant capacity than those expressing a comparable level of wtSOD1. In lowG93A-tTA cells, total GSH after *t*-BHQ treatment was significantly higher than in the highG93A-tTA line and not significantly different from that of highWT-tTA cells (Fig. 8A).

We determined the activity of GCL under the same experimental conditions. *t*-BHQ significantly increased GCL activity only in highWT-tTA cells (Fig. 8B).

Discussion

In the context of evidence of oxidative damage to motor neurons typical of SALS and FALS [1], this study focused on the effects of wild-type and G93ASOD1 on GSH and GCL in an *in vitro* model for FALS1. This is an important data because a

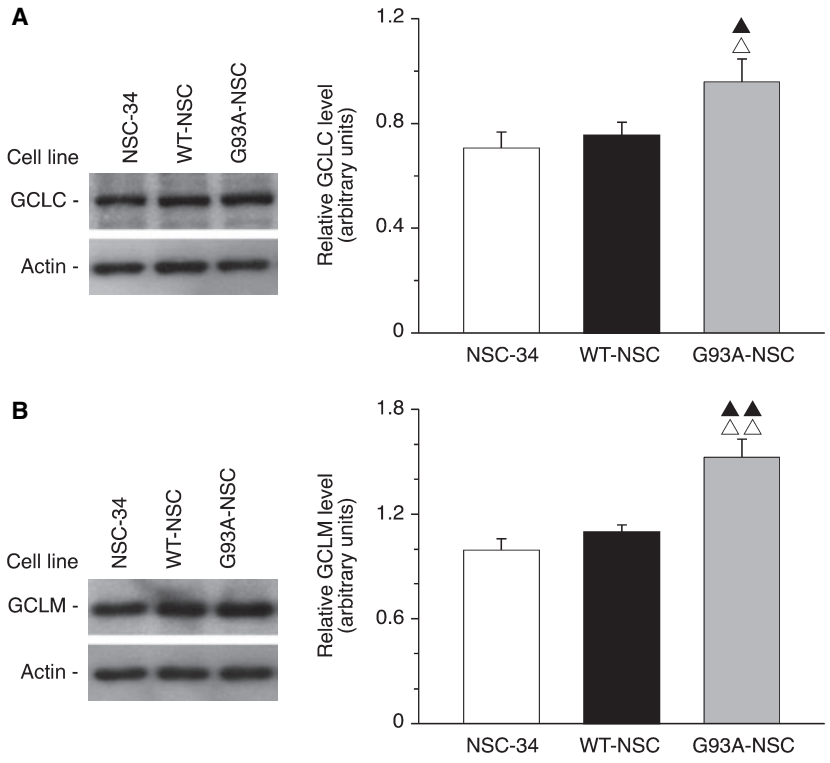

Fig. 6. Expression of GCLC and GCLM in the constitutive FALS1 model. (A) GCLC and (B) GCLM expression of the NSC-34, WT-NSC, G93A-NSC cell lines at their fourth passage. A representative western blot is shown for each protein. The histograms show GCLC and GCLM levels normalized for actin. Values are given as mean ± SEM of four independent experiments. $\Delta P < 0.05$, $\Delta\Delta P < 0.01$ versus NSC-34. $\blacktriangle P < 0.05$, $\blacktriangle\blacktriangle P < 0.01$ versus WT-NSC (one-way ANOVA with Newman–Keuls multiple comparison post-test).

primary decrease in GCL activity causing GSH to decrease might be sufficient to cause spontaneous neuronal death [23].

In motor neuronal cells expressing a low mutant SOD1 content, the response led to increased GSH and GCL activity. By contrast, with high levels of mutant protein, a condition of subtle chronic GSH depletion was established in comparison with controls or wtSOD1 cells. These results highlighted the role of the level of mutant protein in the response of the GSH pathway. In agreement with this, in transgenic G93ASOD1 mice, expressing very high levels of mutant protein, a decrease in mRNA levels of both GCL subunits in the spinal cord was reported as early as at the embryonic stage [24]. In this mouse model, the decrease in GSH in the spinal cord might account, at least in part, for the toxicity of the mutant forms of SOD1 [17]. However, transgenic mice, the unique *in vivo* model available to test the effect of potential therapies, differ from FALS1 patients in terms of the expression level of mutant SOD1 because this is much higher than in patients. Taking into account the results of our *in vitro* model, it is tempting to suggest that the different effects on the GSH pool and/or synthesis accompanying different G93ASOD1 levels might underlie some of the differences existing between the mouse models and patients, for example, in response to some of the therapies that have been tested [25,26]. This might apply in particular to therapies with antioxidants, which may behave differently in the context of altered redox regulation or oxidative stress [27]. Different antioxidants are available which may also act as GSH precursors or not. Our preliminary data suggest that the level of total GSH after acute treatment with *N*-acetylcysteine is modulated by the level and type of SOD1, either wild-type or G93A, whereas it is not influenced by vitamin E (S. Tartari and L. Cantoni, unpublished results).

Our model appears to also provide a tool to investigate the effects of chronic exposure to a small amount of G93ASOD1, as seen in the motor neurons of FALS1 patients. To explain the different amounts of GSH in cells with varying levels of G93ASOD1, we provide evidence of an effect on the expression level of the GCL subunits GCLM and GCLC.

These two subunits contribute differently to the formation of γ-glutamylcysteine, the precursor of GSH. GCLC possesses the catalytic capacity for γ-glutamylcysteine synthesis [28] and its upregulation supports high levels of GSH [23,29].

In our FALS1 models, GCLC increased in the G93A-NSC and lowG93A-tTA cells at the first time point. This might represent the initial response of cells expressing a low level of G93ASOD1, which is possibly more complex because cell homeostasis is less compromised, as suggested by the induction of GR

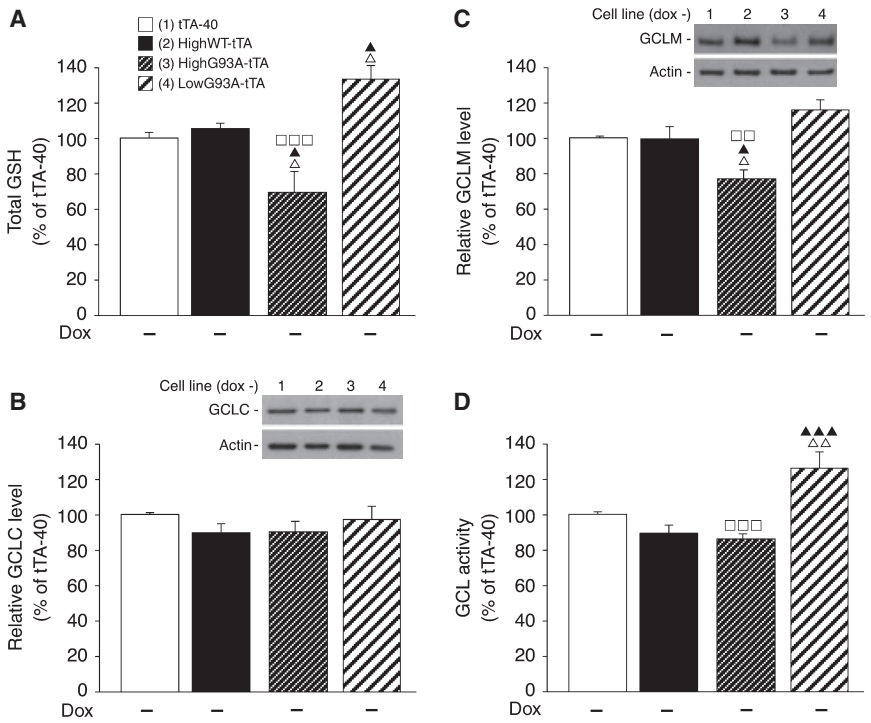

Fig. 7. Effect of time on GSH, GCL activity, GCLC and GCLM expression in the conditional FALS1 model. (A) Total GSH levels of tTA-40, highWT-tTA, highG93A-tTA and lowG93A-tTA cells at the 14th passage. The total GSH level of the tTA-40 cell line (6.73 ± 0.291 $\mu g \cdot mg^{-1}$ of protein) was taken as 100%. Values are given as mean ± SEM of five independent experiments. (B) GCL activity was measured as in (A). The value of the tTA-40 cell line (12.13 ± 0.218 $nmol \cdot min^{-1} \cdot mg^{-1}$ protein) was taken as 100%. Histograms present the mean ± SEM of six independent experiments. (C) GCLC and (D) GCLM expression of the conditional cell lines at the 14th passage. A representative western blot is shown for each protein. GCLC and GCLM levels were normalized for actin. The values of the tTA-40 cell line were taken as 100%. Values are given as mean ± SEM of three independent experiments. $\triangle P < 0.05$, $\triangle\triangle P < 0.01$ versus tTA-40; $\blacktriangle P < 0.05$, $\blacktriangle\blacktriangle\blacktriangle P < 0.001$ versus highWT-tTA; $\square\square P < 0.01$, $\square\square\square P < 0.001$ versus lowG93A-tTA (one-way ANOVA with Newman–Keuls multiple comparison post-test).

and the lack of a decrease in GST. However, the effect of longer exposure of cells to even a low level of mutant protein, studied in the conditional model, was to cancel induction of GCLC, eliminating a factor contributing to the increase in GSH level and GCL activity.

GCLM greatly improves the catalytic efficiency of the holoenzyme GCL [30]. The amount of GCLM is usually lower than the amount of GCLC and limits GSH synthesis [31,32]. Accordingly, there are experimental models showing that overexpression of GCLM increased GSH [29,33], whereas knocking down GCLM lowered it [23,31].

In our model, G93ASOD1 overexpression appeared to affect GCLM more than GCLC. The effects of these modifications are in agreement with reports from the literature on the role of GCLM because the increase in GCLM seemed a convenient way for the G93ASOD1 cells to increase their GSH, whereas the decrease in this subunit – as in highG93A-tTA cells with prolonged exposure to the mutant protein – was concomitant with a decrease in GSH.

This sequential inducing/inhibitory effect of G93ASOD1 on the levels of GCLM and GSH might markedly influence the toxicity of mutant SOD1. In another cell model for FALS1, the high GSH level afforded protection against *S*-nitroso-glutathione toxicity and this was abolished by blocking GSH synthesis [34]. Although GCLM is not essential for viability [31], in contrast to GCLC [35], the lack or disruption of GCLM alone was sufficient to increase cell susceptibility to oxidative stress and nitric oxide [23,31,36], whereas its overexpression rendered cells resistant to oxidative stress [33]. Neurons are especially vulnerable to nitric oxide-mediated mitochondrial damage and neurotoxicity [37,38], and in ALS there is ample evidence that nitric oxide is involved in motor neuron degeneration [39,40]. The increase in GSH also appears essential for adaptation to ER stress [41], which was associated with G93ASOD1 toxicity [42].

A major function attributed to GCLM is to improve the GSH synthesis capacity of the cells [31,32] and this correlates with resistance/recovery from an oxidative

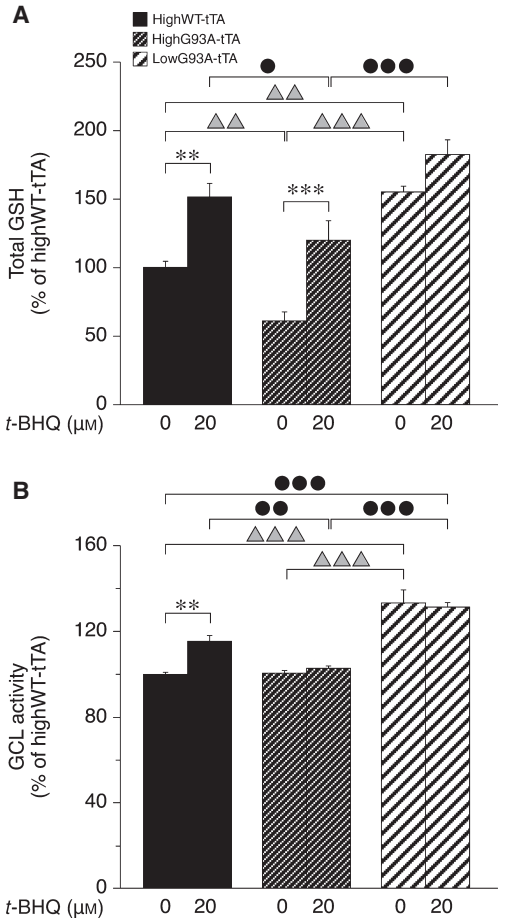

Fig. 8. Effect of *t*-BHQ on GSH and GCL activity in the conditional FALS1 model. The highWT-tTA, highG93A-tTA and lowG93A-tTA cell lines were compared for their response to *t*-BHQ (20 μM). (A) Total GSH and (B) GCL activity were determined 24 h after treatment. No overt toxicity was observed. Cells grown in flasks for 6 days before treatment were at their 14th passage. Results are shown as percentages of the untreated highWT-tTA cells (5.68 $\mu g \cdot mg^{-1}$ protein for total GSH; 12.69 $nmol \cdot min^{-1} \cdot mg^{-1}$ protein for GCL activity). Values are given as mean ± SEM of six independent experiments. For both parameters, statistical significance of differences was assessed by one-way ANOVA with Newman–Keuls multiple comparison post test, comparing the basal levels of the various cell lines (△△$P < 0.01$, △△△$P < 0.001$) or the effect of *t*-BHQ in each cell line (**$P < 0.01$, ***$P < 0.001$) and in the different cell lines (●$P < 0.05$, ●●$P < 0.01$, ●●●$P < 0.001$).

insult even more than GSH level *per se* [43,44]. In our study, GCL activity was not increased in G93ASOD1 cells after *t*-BHQ. In lowG93A-tTA cells, this effect might be explained by the high basal GCL activity [45], whereas in highG93A-tTA cells it suggests a failure of *t*-BHQ to induce GCL. The increase in GSH after *t*-BHQ in this latter cell line may derive from a combination of cytoprotective effects of this treatment [36], however, the increase in highG93A-tTA cells was not comparable with that in highWT-tTA cells. This result reproduced the effect of *t*-BHQ on GSH in cells lacking GCLM [36], further suggesting that the decrease in GCLM in highG93A-tTA cells might play a primary role in the differing toxicity of G93ASOD1 and wtSOD1.

As long as GCL activity and GCLM are elevated, as in lowG93A-tTA cells, motor neuronal cells maintain some antioxidant capacity. For all these reasons, defining the mechanism(s) governing the response of GCLC and GCLM to G93ASOD1 might offer some therapeutic possibilities.

In highWT-tTA cells, the increase in GSH at the early time point may have represented the transient adaptation of cells to the overexpression of wtSOD1 [46], a contributing factor perhaps being the expression of a human protein in a murine cell line. Higher than normal levels of wtSOD1 can alter ROS homeostasis [47], a stimulus that can increase GSH [48]. At least at the level of expression of wtSOD1 in our cells, this increase was not accompanied by significant changes in GCLC and GCLM or GCL activity, and may result from a broad spectrum of changes including the activation of other enzymatic activities [49]. Factors that stimulate cysteine uptake or attenuate GSH feedback inhibition [9] would generally boost the intracellular GSH concentration and might also have a role at the late time point when the total GSH level was higher in highWT-tTA cells than in highG93A-tTA cells. These mechanisms need to be investigated further.

The increase in GSH was long-lasting in lowG93A-tTA cells, coupled with higher GCL activity. In addition to the increased expression of GCL subunits, the GCL activity can also be affected by phosphorylation or nitrosation [9]. Inducers of GCL subunits are environmental or endogenous compounds that cause oxidative stress, but also other stresses [8,22,50,51]. Mutant forms of SOD1 are believed to have aberrant oxidative activities [4]. We have previously reported an increase in ROS formation under basal conditions in the G93A-NSC cells over controls and WT-NSC cells [19]. In this study, induction of GR activity in lowG93A-tTA cells, and the shift to a higher GSH/GSSG ratio in highG93A-tTA cells suggest chronic oxidative stress in cells expressing the mutant protein [6,7]. However, our experimental evidence argues against a mechanism simply implying that increased oxidation of GSH relative to the whole cell is the signal triggering GSH induction, but rather suggests more subtle roles for oxidant species potentially formed in G93A-tTA cells.

The two GCL subunits, GR and GST, are part of the family of the nuclear factor erythroid 2-related fac-

tor 2 (Nrf2)-regulated phase II detoxification enzymes and their regulatory sequence is the anti-oxidant response element (also known as electrophile-response element) [52]. The lack of an increase in GCLC and the decreases in GCLM, GST and GR in highG93A-tTA cells are in agreement with the deficiency in Nrf2-regulated genes in motor neurons from ALS patients and in experimental models of FALS1 [24,53], although the molecular mechanisms behind this finding are yet to be defined. Our results indicated that the enzymes were downregulated with different time courses, suggesting a fine-tuning of their dependency on Nrf2. Nrf2 is a redox-sensitive transcription factor [52]. Induction of GST activity appears to be coupled to a shift in $E_{hGSH/GSSG}$ towards a more oxidized value [54], whereas in highG93A-tTA cells the opposite tendency corresponded to a decrease in GST activity. Studies are now underway in our laboratory to assess the functional links between changes in the redox state of GSH/GSSG and the expression of GST and GCL subunits in G93ASOD1cells. In conclusion, this study provides new information in the field of antioxidant status in ALS, which might be useful in designing effective therapies.

Materials and methods

Materials

The following materials and reagents were used: flasks and plates (Corning Inc., Corning, NY, USA); opti-MEM reduced serum medium, LipofectAMINE 2000, geneticin (G418 sulfate) and hygromycin B (Invitrogen Life Technologies, Paisley, UK); high-glucose Dulbecco's modified Eagle's medium (Cambrex, Verviers, Belgium); fetal bovine serum (Hyclone, Logan, UT, USA); tet-screened fetal bovine serum, pTK-Hyg and pBI-EGFP (Clontech, Palo Alto, CA, USA). All other chemicals and enzymes were purchased from Sigma-Aldrich (St Louis, MO, USA) and Roche (Mannheim, Germany).

Constitutive FALS1 model

The NSC-34 cell line (a kind gift from N. R. Cashman, University of British Columbia, Vancouver, Canada) was used to obtain lines stably expressing human wtSOD1 (WT-NSC) or G93ASOD1 (G93A-NSC) [19].

NSC-34 cells were grown in high-glucose Dulbecco's modified Eagle's medium supplemented with 5% heat-inactivated fetal bovine serum, 1 mM glutamine, 1 mM pyruvate and antibiotics (100 IU·mL^{-1} penicillin and 100 μg·mL^{-1} streptomycin). WT-NSC and G93A-NSC cell lines were maintained in the presence of 0.5 mg·mL^{-1} G418. The cell lines were subcultured in parallel every 7 days so they were all at the same passage number for the experiments.

Conditional FALS1 model

From the NSC-34 cells we obtained the NSC-34 tTA-40 (tTA-40) cell line stably expressing the tetracycline-controlled transactivator protein tTA and permitting tetracycline-regulated gene expression [55]. In our tet-off system, expression of the responsive protein is repressed by the addition of the tetracycline analogue dox to the culture medium. tTA-40 cells were stably co-transfected, following the LipofectAMINE 2000 reagent protocol with pBI-EGFP containing human wild-type or G93ASOD1 cDNA and pTK-Hyg to obtain conditional clones (WT-tTA and G93A-tTA) expressing hygromycin resistance and the two forms of SOD1 [21,55]. Multiple WT-tTA or G93A-tTA clones were isolated after 4 weeks' selection with hygromycin B (0.2 mg·mL^{-1}) and maintained in culture with dox (2 μg·mL^{-1}). Cells of each clone were detached using NaCl/P_i–EDTA, pelletted by centrifugation, washed again with NaCl/P_i while in suspension and plated with or without dox (dox+/dox−) in the culture medium. After 48 h, when the medium was changed, dox− cells were again washed with NaCl/P_i to remove dox released by cells and allow rapid transgene expression [56]. All cells were collected 96 h after plating and screened by western blot for the level of the transfected SOD1 in dox+/dox− culture conditions. After this screening, only dox+ cultured cells were stored in liquid nitrogen. The following cell lines were used: tTA-40 (control), cells with a high level of wtSOD1 (highWT-tTA) and cells with a high or a low level of G93ASOD1 (high and lowG93A-tTA respectively).

tTA-40 cells were cultured in the same way as NSC-34 cells except that tet-screened heat-inactivated fetal bovine serum (5%) was used and G418 sulfate (0.5 mg·mL^{-1}) was added. Hygromycin (0.2 mg·mL^{-1}) was added to the medium for WT-tTA and G93A-tTA cells. In the dox+ culture 1 μg·mL^{-1} dox was added every 2 days while changing the culture medium.

Samples for the determination of GSH, SOD1 and GCL subunit levels and GCL activity

Samples of the conditional cell lines were thawed (time 0) and cultured with dox (Fig. 1A). At the end of the second week of culture (second passage), each cell line was split into two flasks, which were then cultured in parallel so that they were all at the same passage number for the experiments. One flask continued receiving dox (dox+), whereas in the other dox was removed (dox−) using the procedure described above, to allow full expression of the transfected SOD1. In the dox− cells SOD1 was fully expressed from 96 h after the second passage (Fig. 1A). Cells were collected

at the fourth passage, corresponding to 4 weeks' culture, for analysis relative to the first time point and at the 14th passage. The growth curves of the conditional cell lines did not significantly differ (data not shown).

NSC-34, WT-NSC and G93A-NSC cell lines were thawed and cultured under standard conditions. Cells were collected after 4 weeks' culture (fourth passage). As previously reported, these cell lines did not differ in their proliferation [19].

GSH measurements

Seven days before each selected time point, cells (plated at 6850 cells·cm^{-2} in T25 flasks) were allowed to grow under standard conditions (dox−/dox+ for the conditional cell lines). Cells were collected and washed twice by centrifugation with Dulbecco's NaCl/P_i; the final pellet was resuspended with 5% sulfosalicylic acid (120 μL), incubated for 1 h on ice and centrifuged at 14 000 ***g*** for 10 min. The supernatant was used to determine total GSH and GSSG following the 5,5′-dithiobis (2-nitrobenzoic acid) GR recycling assay [57]. Total GSH was measured spectrophotometrically at 30 °C as GSH equivalents (GSH + 2 GSSG). Supernatant (25 μL) was added to an assay mixture consisting of 0.7 mL NADPH (0.3 mM) dissolved in sodium phosphate (125 mM), pH 7.5, containing EDTA (6.3 mM), 0.1 mL 5,5′-dithiobis (2-nitrobenzoic acid) (6 mM) dissolved in sodium phosphate/EDTA and water to 1 mL. After 2 min preincubation, 0.6 U GR was added to the 1-mL assay mixture and the change in absorbance at 412 nm was measured over 3 min. Standard curves were generated using GSH solutions in 5% sulfosalicylic acid.

To measure GSSG, 70 μL of supernatant was mixed with 4.2 μL of triethanolamine and 1.4 μL of 2-vinylpyridine, which reacts with GSH masking it to GR, and the reaction mixture was incubated at room temperature for 60 min. Samples were then assayed as described above for total GSH, but with double the amount of GR. Standard curves were generated with GSSG solutions and the addition of 2-vinylpyridine to the assay mixture. GSSG concentrations in the cell extracts were in the middle of the range of the standard curve (0–0.20 pmol). The amount of GSH was calculated by subtracting twice the amount of GSSG from the total.

The protein pellet was resuspended in 1 M NaOH and used to determine the protein content of the sample with a bicinchoninic acid assay kit (Pierce, Rockford, IL, USA) to normalize values for total GSH, GSH and GSSG.

GSH : GSSG ratio and $E_{hGSH/GSSG}$ in the conditional cell lines

Two different parameters were used to indicate the redox state of the cell [7,54]. The first was the ratio of GSH to GSSG, which takes into account especially mechanisms of *S*-thiylation for protein control. The second was the reduction potential of the GSH/GSSG couple ($E_{hGSH/GSSG}$), which takes into account mechanisms of oxidation reduction of dithiol motifs for protein control, calculated using the Nernst equation as described by Jones [58] and Halvey *et al.* [59]. Redox potentials are presented as millivolts (mV).

SDS/PAGE and western blot

To analyse SOD1 expression, cells grown in T25 flasks were collected and washed with Dulbecco's NaCl/P_i. The cell pellet was lysed for 10 min at 4 °C in 50 mM Tris/HCl (pH 8.0) containing 150 mM NaCl, 1% SDS and a protease inhibitor cocktail (Sigma-Aldrich). The sample was then boiled at 95 °C for 5 min and a whole-cell lysate was obtained. The procedure described by Diaz-Hernandez *et al.* [23] was used to analyse GCLC and GCLM expression. Cells were lysed for 20 min at 4 °C in Tris/HCl (20 mM, pH 8.0), containing 1% Nonidet-P40, 5 mM EDTA, 2 mM EGTA, 137 mM NaCl, 10% glycerol, 1 mM Na_3VO_4, 50 mM NaF and a protease inhibitor cocktail (Sigma-Aldrich). Extracts were centrifuged at 13 000 ***g*** for 20 min at 4 °C and aliquots of the supernatant were used.

Proteins (10–30 μg) were separated by electrophoresis on 12% or 10% polyacrylamide gels, respectively for SOD1 or GCLC and GCLM determination. Nitrocellulose membranes were probed with the following primary antibodies: humanSOD1 (sheep polyclonal; Calbiochem, EMD Biosciences, Inc. La Jolla, CA, USA), actin (mouse monoclonal; Chemicon International Inc., Temecula, CA, USA) [19,55], GCLC (1 : 1600; rabbit polyclonal, Lab Vision Corporation, Fremont, CA, USA) or GCLM (1 : 10 000; rabbit polyclonal, a kind gift from T. J. Kavanagh, University of Washington, Seattle, WA, USA). GCLC and GCLM antibodies were used coupled to a secondary antibody to rabbit raised in goat (1 : 2000). Protein bands were detected with the ECL detection system (Amersham Biosciences, Little Chalfont, UK). Films were scanned and band intensities obtained with an AIS Image Analyser (Imaging Research Inc., St Catharine's, Canada).

Enzymatic activities

Cells (plated in T25 flasks, 6850 cells·cm^{-2}) were allowed to grow for 7 days under standard conditions. Cells were then collected and washed twice by centrifugation with Dulbecco's NaCl/P_i; the final pellet from each flask was resuspended in 0.55 mL of buffer (50 mM potassium phosphate, pH 7.5, with 1 mM EDTA), sonicated and centrifuged at 12 000 ***g*** for 30 min. The supernatants were used to measure the enzymatic activities after determining the protein content with the bicinchoninic acid assay.

GCL activity was determined as described by Zhou & Freed [60]. The reaction mixture (final volume 200 μL) contained 100 mM Tris/HCl (pH 8.2), 20 mM $MgCl_2$, 150 mM KCl, 10 mM L-glutamate, 10 mM L-cysteine, 5 mM ATP,

2 mM EDTA, 0.2 mM NADH, 2 mM phosphoenolpyruvate, pyruvate kinase (2 U) and lactate dehydrogenase (2 U). The reaction was started by adding 100 μg protein and the decrease in absorbance at 340 nm in a 96-well plate was followed for 5 min at 25 °C. Specific activity was expressed in $U \cdot mg^{-1}$ protein and then as a percentage of control.

GST activity was measured as described by Habig *et al.* [61]. The reaction mixture (final volume 300 μL) contained 100 mM potassium phosphate (pH 6.5), 1 mM EDTA, 1 mM 1-chloro-2,4-dinitrobenzene and 2 mM GSH. The reaction was started by adding 30 μg of protein. The increase in absorbance at 340 nm in a 96-well plate was followed for 5 min at 25 °C after 5 min preincubation.

GR activity was measured as described by Allen *et al.* [13] except that the concentration of GSSG was 1 mM. The other components of the reaction mixture (final volume 300 μL) were: 50 mM Hepes/KOH (pH 8.0), 0.1 mM EDTA and 30 μg of protein. The reaction was started by adding NADPH (0.1 mM). The decrease in absorbance at 340 nm was followed for 5 min at 25 °C in a 96-well plate after 5 min preincubation.

Treatment with *tert*-butylhydroquinone (*t*-BHQ)

Cells (6850 $cells \cdot cm^{-2}$) were grown under standard dox– conditions in T25 flasks for 6 days and then treated with *t*-BHQ (20 μM final concentration) for 24 h.

Statistical analysis

One-way analysis of variance (ANOVA), followed by Newman–Keuls multiple comparison post-test was used for statistical analysis.

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Supporting information

The following supplementary material is available:
Fig. S1. Effect of different times of exposure to doxycycline (dox) on expression of enhanced green fluorescent protein (EGFP) and Cu,Zn superoxide dismutase (SOD1).
Doc. S1. Additional method. Repression of Cu,Zn superoxide dismutase (SOD1) and enhanced green fluorescent protein (EGFP) expression by doxycycline (dox).

This supplementary material can be found in the online version of this article.

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