Centre de Neurosciences Psychiatriques

GLUTATHIONE DEFICIT IN SCHIZOPHRENIA: STRATEGIES TO INCREASE GLUTATHIONE LEVELS IN *IN VITRO* AND CLINICAL STUDIES

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2. Summary

Decrease in glutathione (GSH) levels was observed in cerebrospinal fluid, prefrontal cortex and post-mortem striatum of schizophrenia patients. Evidences suggest a defect in GSH synthesis at the levels of the rate-limiting synthesizing enzyme, glutamate cysteine ligase (GCL). Indeed, polymorphisms in the gene of the modifier subunit of GCL (GCLM) was shown to be associated with the disease in three different populations, GCLM gene expression is decreased in fibroblasts from patients and the increase in GCL activity induced by an oxidative stress is lower in patients' fibroblasts compared to controls. GSH being a major antioxydant and redox regulator, its presence is of high importance for protecting cells against oxidative stress. The aim of the present work was to use various substances to increase GSH levels by diverse strategies. Since the synthesizing enzyme GCL is defective, bypassing this enzyme was the first strategy we used. GSH ethyl ester (GSHEE), a membrane permeable analog of GSH, succeeded in replenishing GSH levels in cultured neurons and astrocytes previously depleted in GSH by L-buthionine-(S,R)-sulfoximine (BSO), an inhibitor of GCL. GSHEE also abolished dopamine-induced decrease of NMDA-mediated calcium response observed in BSO-treated neurons. γ-Glutamylcysteine ethyl ester (GCEE), a membrane permeable analog of the product of GCL, increased GSH levels only in astrocytes. The second strategy was to boost the defective enzyme GCL. While quercetin (flavonoid) could increase GSH levels only in astrocytes, curcumin (polyphenol) and *tert*-butylhydroquinone (quinone) were successful in both neurons and astrocytes, via an increase in the gene expression of the two subunits of GCL and, consequently, an increase in the activity of the enzyme. However, FK506, an immunosupressant, was unefficient. Treating astrocytes from GCLM KO mice showed that the modulatory subunit is necessary for the action of the substances. Finally, since cysteine is the limiting precursor in the synthesis of GSH, we hypothesized that we could increase GSH levels by providing more of this precursor. N-acetyl-cysteine (NAC), a cysteine donor, was administered to schizophrenia patients, using a double-blind and cross-over protocol. NAC significantly improved the mismatch negativity (MMN), a component of the auditory evoked potentials, thought to reflect selective current flowing through open, unblocked NMDA channels. Considering that NMDA function is reduced when GSH levels are low, increasing these levels with NAC could improve NMDA function as reflected by the improvement in the generation of the MMN.

3. Résumé

Les taux de glutathion (GSH) dans le liquide céphalo-rachidien, le cortex préfrontal ainsi que le striatum post-mortem de patients schizophrènes, sont diminués. L'enzyme limitante dans la synthèse du GSH, la *glutamyl-cysteine ligase* (GCL), est défectueuse. En effet, des polymorphismes dans le gène de la sous-unité modulatrice de GCL (GCLM) sont associés à la maladie, l'expression du gène GCLM est diminuée dans les fibroblastes de patients et, lors d'un stress oxidative, l'augmentation de l'activité de GCL est plus faible chez les patients que chez les contrôles. Le GSH étant un important antioxydant et régulateur du status redox, sa présence est primordiale afin de protéger les cellules contre les stress oxydatifs. Au cours du présent travail, une variété de substances ont été utilisées dans le but d'augmenter les taux de GSH. Passer outre l'enzyme de synthèse GCL qui est défectueuse fut la première stratégie utilisée. L'éthyl-ester de GSH (GSHEE), un analogue du GSH qui pénètre la membrane cellulaire, a augmenté les taux de GSH dans des neurones et des astrocytes déficitaires en GSH dû au L-buthionine-(S,R)-sulfoximine (BSO), un inhibiteur du GCL. Dans ces neurones, le GSHEE a aussi aboli la diminution de la réponse NMDA, induite par la dopamine. L'éthyl-ester de γ-glutamylcysteine (GCEE), un analogue du produit de la GCL qui pénètre la membrane cellulaire, a augmenté les taux de GSH seulement dans les astrocytes. La seconde stratégie était d'augmenter l'activité de l'enzyme GCL. Tandis que la quercétine (flavonoïde) n'a pu augmenter les taux de GSH que dans les astrocytes, la curcumin (polyphénol) et le *tert*-butylhydroquinone (quinone) furent efficaces dans les deux types de cellules, via une augmentation de l'expression des genes des deux sous-unités de GCL et de l'activité de l'enzyme. Le FK506 (immunosupresseur) n'a démontré aucune efficacité. Traiter des astrocytes provenant de souris GCLM KO a permis d'observer que la sous-unité modulatoire est nécessaire à l'action des substances. Enfin, puisque la cysteine est le substrat limitant dans la synthèse du GSH, fournir plus de ce présurseur pourrait augmenter les taux de GSH. N-acétyl-cystéine (NAC), un donneur de cystéine, a été administrée à des schizophrènes, lors d'une étude en double-aveugle et *cross-over*. NAC a amélioré le *mismatch negativity* (MMN), un composant des potentials évoqués auditifs, qui reflète le courant circulant via les canaux NMDA. Puisque la fonctionnalité des R-NMDA est diminuée lorsque les taux de GSH sont bas, augmenter ces taux avec NAC pourrait améliorer la fonction des R-NMDA, réflété par une augmentation de l'amplitude du MMN.

4. Introduction

4.1 Schizophrenia

Schizophrenia is a severe mental disorder that affects about seven people out of a thousand in the worldwide adult population (Mental Health Organization Website). The term "schizophrenia" was proposed by a Swiss psychiatrist named Eugène Bleuler. In 1911, Bleuler used Greek roots to build the word that, according to him, illustrated the best the division between certain psychic functions. From an etymological point of view, "schizo" or *skhizein* means to split and "phrenia" or *phrên*, means mind. This disease is characterized by impressive symptoms, named "positives", such as hallucinations, disorganized speech and behavior, delirium and paranoia. More discrete symptoms, the negative ones, like depression, apathy and language poverty are also present. At last, another important component common to patients suffering from schizophrenia includes cognitive symptoms, such as attention, planning, memory and executive functions deficits, and basic symptoms like perceptual instability. Schizophrenia is a heterogeneous disease and the manifestation of these symptoms is highly variable from a patient to another.

Since schizophrenia was identified, medicine doctors have worked on the identification of symptoms, in order to better classify the disease. As for neuroscientists, they have for mission to understand the "how" of that complex disorder. The goal of these two professions is the same: finding a more efficient treatment in order to lighten the burden of that mentally, but also socially devastating disease. Many observations suggest the presence of a strong genetic component in the susceptibility to schizophrenia (Karayiorgou and Gogos, 1997;Lewis and Lieberman, 2000). First, family studies demonstrated that an individual's lifetime risk of developing schizophrenia increases exponentially with degree of relation to an affected individual. Second, twin studies comparing the concordance for schizophrenia in monozygotic (MZ) twin pairs (who, in principle, share all of their genes) with the concordance in dizygotic (DZ) twin pairs (who share half of their genes) demonstrated that the MZ concordance rate (46%–48%) is significantly higher than the DZ rate (4%–14%). Finally, adoption studies comparing the prevalence of schizophrenia in biological relatives who have been separated by adoption early in life and in adoptive, not genetically related relatives who shared the same environment, demonstrated that the prevalence of schizophrenia is higher in the

biological than in the adoptive relatives. Linkage studies allowed the identification of various susceptible genes such as proline dehydrogenase (PRODH), neuregulin 1 (NRG1), disrupted in schizophrenia 1 (DISC1; see Gogos and Gerber, 2006 for a more exhaustive list). Some other genes, such as catechol-O-methyltransferase (COMT), regulator of G-protein signaling 4 (RGS4) and calcineurin γ catalytic subunit were rather identified through candidate gene approaches (Gogos and Gerber, 2006). On the other hand, the significant discordance between identical twins points towards the importance of environmental factors. Probable environmental insults may include viral infections (Leweke et al., 2004), autoimmune disorders, exposure to adverse intrauterine events, and obstetric complications or birth traumas (Cannon et al., 2000;Rosso et al., 2000).

Because of the complexity of schizophrenia, the causes of this mental disorder are still not well understood. For more than five decades, various hypotheses trying to explain the causes of schizophrenia have followed or crosschecked one another. The most popular of the proposed hypotheses was the hyperactivity of dopamine transmission. This was based on the observations that antagonists of dopamine D2 receptors could improve positive symptoms and that dopamine-enhancing drugs show psychotogenetic effects (Laruelle et al., 2005). More recently, studies have shown direct evidence of dopamine abnormalities at the level of storage, vesicular transport, release, or reuptake by the presynaptic neuron (Lewis and Lieberman, 2000). Then, in the ‘90s, several studies have demonstrated that the administration of NMDA-R antagonists, such as phencyclidine (PCP) or ketamine, to control subjects lead to the apparition of symptoms similar to those observed in schizophrenia and exacerbates these symptoms in patients (Krystal et al., 1994). The ability of both noncompetitive and competitive NMDA-R antagonists to induce schizophrenia-like psychotic symptoms and cognitive impairments may indicate that endogenous dysfunction or dysregulation of NMDA-R-mediated neurotransmission is critically implicated in the pathophysiology of schizophrenia (Javitt and Zukin, 1991). Also, multiple roles of the NMDA-R in regulating neuronal migration, neuronal differentiation, response to trophic factors, functional plasticity such as long-term potentiation and long-term depression, and finally the elaboration of synaptic spines, are consistent with the multiple functional and structural abnormalities documented in schizophrenia. Therefore, many therapeutic strategies are focusing on enhancing NMDA-R function. Indeed, treatments with

NMDA agonists such as glycine (Heresco-Levy et al., 1999;Javitt et al., 1994) and D-serine (Tsai et al., 1998) provide symptoms improvement in schizophrenia. The dopamine and glutamatergic systems have received the most attention, although other systems, such as GABAergic, serotoninergic and opioid, have also been implicated. In addition, evidences indicate that oxidative stress is associated with schizophrenia patients (Mahadik et al., 1998;Reddy et al., 2003). It is however not known whether this oxidative stress is due to excess of reactive oxygen species (ROS) or to a deficit in antioxidant mechanisms or to a combination of both. An excess of ROS could originate from an excess of endogenous products, toxic environmental compounds, or pathological insults (such as infections or hypoxia/anoxia). A deficit in antioxidant mechanisms could be due to a genetic defect in one or some enzymes involved in the defense mechanisms such as superoxide dismutase, catalase or those of the glutathione (GSH) metabolism. Impairment of the antioxidant systems might render the organism particularly vulnerable during a temporary excess of ROS. Although the underlying mechanisms remain unclear, our laboratory proposes a genetic defect of the GSH system as a vulnerability factor for schizophrenia (Do et al., 2004;Do et al., 2007, in press).

4.2 Glutathione deficit in schizophrenia

Cerebrospinal fluid (CSF) of 26 patients suffering from schizophrenia and 14 control subjects was collected and analyzed by high-performance liquid chromatography (HPLC) and mass spectroscopy. These techniques allow separating the constituents of a mix in order to quantify them. A decrease in the concentration of GSH (-27%) and of its direct metabolite, g-glutamyl-glutamine (-15%) was observed in drug-naive patients compared to controls (Do et al., 2000). GSH levels were found to be 52% lower in medial prefrontal cortex using *in vivo* technique (Do et al., 2000) and 40% lower in postmortem striatum (Yao et al., 2006) of patients compare to controls. Different lines of evidence suggest a defect in GSH synthesis at the level of the key-synthesizing enzyme, glutamyl-cysteine ligase (GCL). Indeed, polymorphisms in the gene of the modulatory subunit of GCL (GCLM) was shown to be associated with the disease in three different populations and GCLM gene expression is decreased in fibroblasts of patients (Tosic et al., 2006). Finally, the increase in GCL activity induced by an

oxidative stress is lower in patients' fibroblasts compared to controls (Gysin et al., 2005).

Combined with other genetic and with environmental factors such as stress, obstetrical complications or viral infections, a deficit in GSH could favor the development of schizophrenia. The role of GSH deficit proposed allows to integrate in a causal way many phenomenological aspects of schizophrenia. It is compatible with both the DA and the glutamate/NMDA hypotheses and with the neuropathological observations. Life event stresses, through hypothalamic-pituitary-adrenal axis stimulation, induce important DA release that could result, when combined with GSH deficit, in an increase in ROS and thus in oxidative damage to lipid, protein and DNA. This leads, during brain development and maturation, to progressive structural and functional disconnectivity. Moreover, the effect of an acute GSH deficit on the function of NMDA-R and on dopamine signaling indicates that a GSH deficit during adulthood could also contribute to the symptomatology of the illness (Do et al., 2007, in press).

Various observations support the implication of a GSH deficit in the pathophysiology of schizophrenia. Animal models in which GSH levels are transitory decreased and dopamine is increased during development, present morphological and behavioral impairments similar to those observed in schizophrenia: decrease in dendritic spine density in pyramidal neurons of prefrontal cortex (Garey et al., 1998;Gheorghita et al., submitted;Glantz and Lewis, 2000;Kolluri et al., 2005), anomalies of parvalbumine immunoreactive GABA interneurons in anterior cingulate cortex (Cabungcal et al., 2006;Lewis et al., 2005), and impairments in cognitive performance (Cabungcal et al., in press;Castagné et al., 2004a;Castagné et al., 2004b;Robbins, 2005). It was also shown that GSH, as a reducing agent, can potentiate the activity of redox sensitive proteins, such as NMDA-R (Choi and Lipton, 2000;Kohr et al., 1994). In addition, as the cell attempts to prevent damage from oxidative stress, GSH is converted to its oxidized form (GSSG), that is actively transported outside the cell to avoid a shift in the redox equilibrium. This GSSG can accumulate in the extracellular space and lead to a decrease in the activity of NMDA-R (Janaky et al., 1993;Sucher and Lipton, 1991). *In vitro* experiments with an animal models in which GSH levels are decreased, show hypofunction of NMDA-R (Steullet et al., submitted;Steullet et al., 2006). Altogether,

these evidences point towards the hypothesis that the dysregulation of the GSH system could be implicated in the reduced activity of NMDA-R.

4.3 Glutathione

γ-L-glutamyl-L-cysteinylglycine, or glutathione, is a tripeptide made of glutamate, cysteine and glycine (figure 1). This substance was first described in 1888 by de Rey-Pailhade in a report of the Académie des Sciences (see Meister, 1989). Because of its affinity to sulfur, de Rey-Pailhade named this substance *philothion* (from the Greek: *philo* = to love and *thion* = sulfur). We had to wait until 1921 for Hopkins to change the name of the substance to "glutathione" and to describe its reducing properties (Hopkins, 1921).

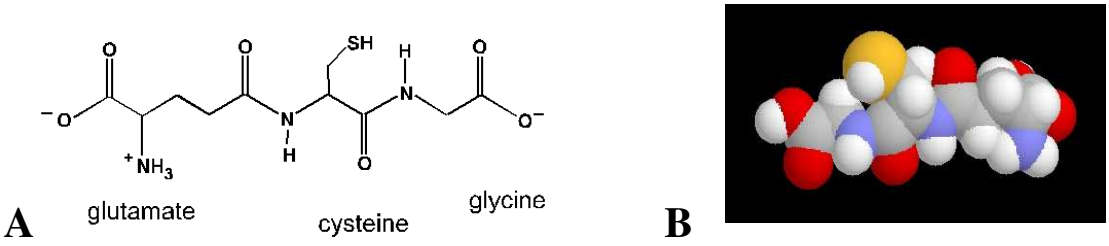


Figure 1. Reduced glutathione (GSH)

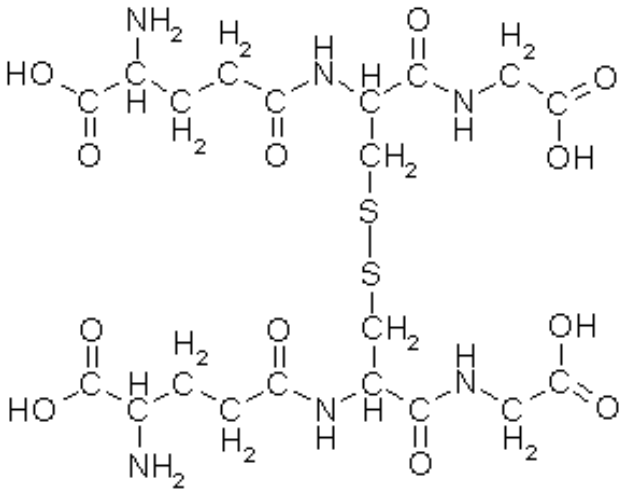


Figure 2. Oxidized glutathione (GSSG)

4.3.1 Redox cycle

Glutathione can be found under its reduced (GSH; figure 1) and oxidized (GSSG; figure 2) forms, the two of them being interchangeable following a redox cycle (figure 3). GSH is the substrate of an enzyme named *GSH peroxidase* (GPx) that reduces hydrogen peroxide (H_2O_2) to produce GSSG and H_2O. *GSH transhydrogenase* allows GSH to act as an electron donor that reduces disulfide bounds found on plasmatic proteins and therefore lead to the formation of new thiols. Finally, GSSG is reduced by the *GSSG reductase*, a reaction requiring the oxidation of one molecule of NADPH.

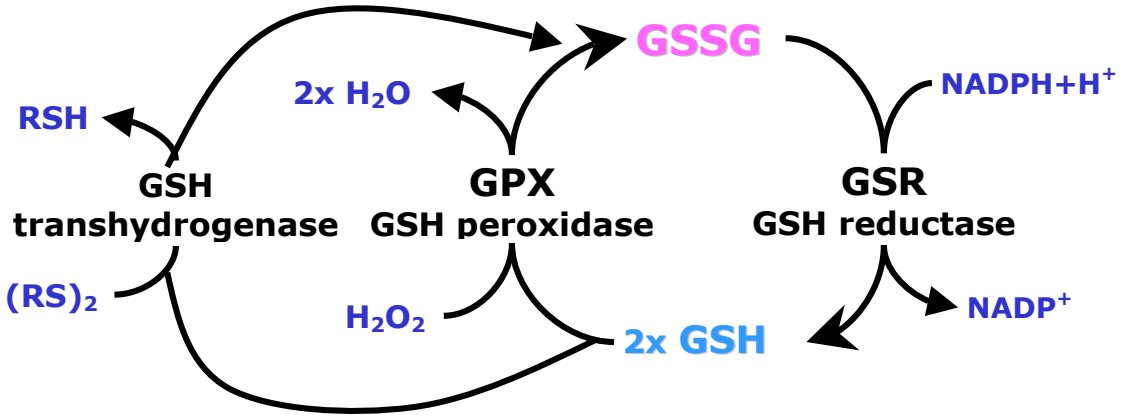


Figure 3. Glutathione redox cycle

4.3.2 Synthesis and metabolism

GSH is synthesized following two reactions (figure 4) catalyzed by the *γ-glutamylcysteine ligase* (GCL) and the *glutathion synthetase* (GSS). The first reaction links glutamate to cysteine to produce γ-glutamylcysteine (γ-glucys), to which glycine is subsequently added during the second reaction. Each enzyme consumes one ATP per catalytic cycle. GCL is a heterodimer made of one catalytic subunit (GCLC), which contains the substrates binding sites, and one modulatory subunit (GCLM). The later is smaller and it modulates the affinity of GCLC for the substrates and inhibitors. GCL, the rate-limiting enzyme in the synthesis of GSH, is feedback-inhibited by GSH (Richman and Meister, 1975). Catabolism of GSH takes place outside the cell and is catalyzed by the *γ-glutamyl transpeptidase* (γGT) that is bound to the external surface of the cell membrane. Transpeptidation requires the presence of a suitable amino acid

(AA; e.g. glycine or methionine) and leads to the formation of γ-glutamyl-AA and cysteinylglycine. The dipeptide cysteinylglycine will be hydrolyzed by dipeptidases to free amino acids that will be recycled to GSH synthesis. GSH can also take the path of the *GSH-S-transferase* (GST), an important detoxifying enzyme that catalyses the conjugation of GSH with various kinds of electrophiles. γ-glutamyl-AA is the substrate of the *γ-glutamylcyclotransferase* that converts it into 5-oxoproline. 5-oxoproline is then converted into glutamate by the enzyme *5-oxoprolinase*, reaction requiring one ATP. This glutamate can then be recycled in the synthesis of new GSH.

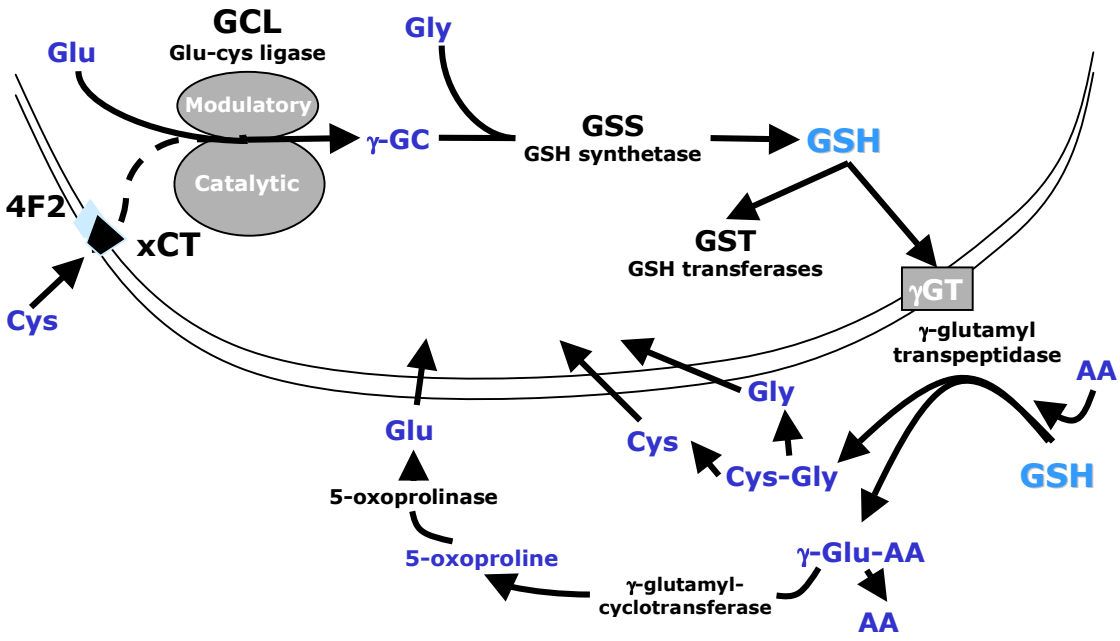


Figure 4. Glutathione synthesis and metabolism

4.4 Roles of glutathione

GSH has several roles in the nervous system (Shaw, 1998), including DNA synthesis and repair, and proteins and prostaglandin synthesis. But the most important of its properties is its faculty to protect cells against oxidative stress by regulating the redox state of the cell, scavenging free radical, participating in the elimination of toxins and acting as a source of cysteine. These roles will be discussed in more details.

4.4.1 Protecting cells against oxidative stress

4.4.1.1 Oxidative stress

Although oxygen is essential for life, highly reactive molecules, collectively termed reactive oxygen species (ROS), are produced during oxidative phosphorylation by mitochondria. ROS are therefore free radical atoms or molecules with unpaired electrons, including superoxide anions ($O_2\bullet^-$), peroxides ($O_2\bullet^{-2}$) and the highly reactive hydroxyl radical (•OH). The main source of ROS *in vivo* is aerobic mitochondrial respiration, although ROS are also produced during normal metabolism activity such as peroxisomal β-oxidation of fatty acids. ROS can also be produced as a consequence of various environmental perturbations such as extreme temperatures, ionizing radiations, xenobiotics, toxins, air pollutants, various biotic and abiotic stresses, and diseases (Scandalios, 2005). ROS are cleared from the cell by the action of the enzymes superoxide dismutase (SOD), leading to the production of hydrogen peroxides (H2O2; Johnson and Giulivi, 2005). These peroxides can be reduced by catalase in peroxisomes, or by glutathione peroxidase (GPx) in the cytoplasm. ROS can also be conjugated with antioxidants, the most important being GSH. When natural defense mechanisms of the organism are efficient, ROS exist in balance with biochemical antioxidants and detoxifying enzymes. When this balance is altered to favor ROS, oxidative stress occurs, resulting in a build-up of oxidatively modified molecules that can disrupt normal cellular activity. Thus, during times of environmental perturbations, ROS levels can increase dramatically, resulting in significant damage to cell structures. The main damage to cells results from the interaction of ROS with other molecules within the cell, which can cause oxidative damage to proteins, membranes and DNA (Halliwell, 1999;Halliwell and Chirico, 1993). The effects of oxidative stress depend upon the size of the changes induced, with a cell being able to overcome small perturbations and regain its original state. However, more severe oxidative stress can cause cell death and even moderate oxidation can trigger the apoptosis, and more intense stresses causing necrosis (Lennon et al., 1991).

Thanks to its thiol group (figure 1B, yellow moiety), GSH can scavenge free radicals, but it can also detoxify the potential harmful xenobiotics and their metabolites. GSH forms S-conjugates with electrophiles in reactions that are catalyzed by GSH-S-

transferases (Hayes et al., 2005). Conjugation reactions usually render electrophilic xenobiotic intermediates less reactive and, in most cases, reduce or eliminate their pharmacological and/or toxic action. These conjugates must then be exported from cells so that they can be eliminated from the body. Very little is known about GSH and GSH-S-conjugates transport outside the cell (Ballatori et al., 2005). A family of transporters named mutidrug resistance proteins (MRP) was demonstrated to mediate the cellular efflux of GSH conjugates and GSH itself (for a review see Cole and Deeley, 2006).

4.4.1.2 Induction of cellular defense system

In order to detoxify xenobiotics or ROS, phase I and II biotransformations are necessary. Phase I reactions may occur by oxidation, reduction or hydrolysis of xenobiotics to electrophilic intermediates. If the metabolites of phase I reactions are sufficiently polar, they may be readily excreted at this point. However, many phase I products are not eliminated rapidly and undergo a subsequent reaction in which an endogenous substrate combines with the newly incorporated functional group to form a highly polar conjugate. These conjugation reactions are known as phase II reactions. Some xenobiotics are sufficiently electrophilic to undergo conjugation without prior modification by phase I enzymes. GSH being a major reducing agent during phase I reactions and an important conjugate in phase II reactions, the utilization of GSH in these essential cellular defense mechanisms results in depletion of GSH pools that ultimately must be replenished. This is done through two major routes, reduction of GSSG via GSH reductase and *de novo* synthesis via GCL and GSS.

It was shown that changes in the activity of GCL come along with changes in GSH levels, suggesting that the regulation of GCL activity is important in the maintenance of an appropriate amount of GSH in the cell (Morales et al., 1997). A great number of substances can induce the transcription of the genes coding for GCLM and/or GCLC. Most of these substances, if not all, are electrophiles, also named Michael reaction acceptors (Talalay et al., 1988). Many of these agents are inducers of phase II detoxifying enzymes and have been reported to initiate a protective response, designated as the “electrophiles counterattack” (Prestera et al., 1993). This response is characterized by the increase in intracellular GSH and the up-regulation of detoxification enzymes. The expression of these genes is regulated by the antioxidant response element (ARE)/electrophile response elements (EpRE) which is a *cis*-acting

regulatory element found in promoter regions of phase II genes (Rushmore et al., 1991). Indeed, ARE/ EpRE have been identified in the promoter regions of GST (Rushmore and Pickett, 1990), NAD(P)H:quinone oxidoreductase (NQO1; Favreau and Pickett, 1991;Friling et al., 1990) and heme-oxigenase 1 (HO-1; Prestera et al., 1995), but also in the genes encoding the catalytic (Mulcahy et al., 1997) and the modulatory (Moinova and Mulcahy, 1998) subunits of GCL.

Nrf2 is a transcription factor that binds to ARE/EpRE elements and activates genes that participate in the defense against free radicals and toxic insults (figure 5). Nrf2 was shown to be essential for the coordinate induction of phase II detoxifying and antioxidant enzymes, both of which are under ARE regulation (Ishii et al., 2000;Itoh et al., 1999). ARE activation signals, such as ROS and electrophiles, dissociate the cytoplasmic Nrf2-Keap1 complex, allowing Nrf2 to translocate into the nucleus (Jaiswal, 2004). Nrf2 heterodimerize with a partner that is still unknown, probably a small Maf, and this complex binds to the ARE and transcriptionally activates downstream target genes (Zhang and Gordon, 2004). Activation proteins (AP)-1 transcription factors including Jun and Fos also join the complex, but their role, as well as that of small Mafs is still unclear. They seem to negatively or positively regulate ARE-mediated gene expression depending on cell types and genes, presumably to keep the expression of antioxidant enzymes ‘‘in check’’, to maintain the cellular defenses active and/or to rapidly restore induced enzymes to normal levels (Jaiswal, 2004).

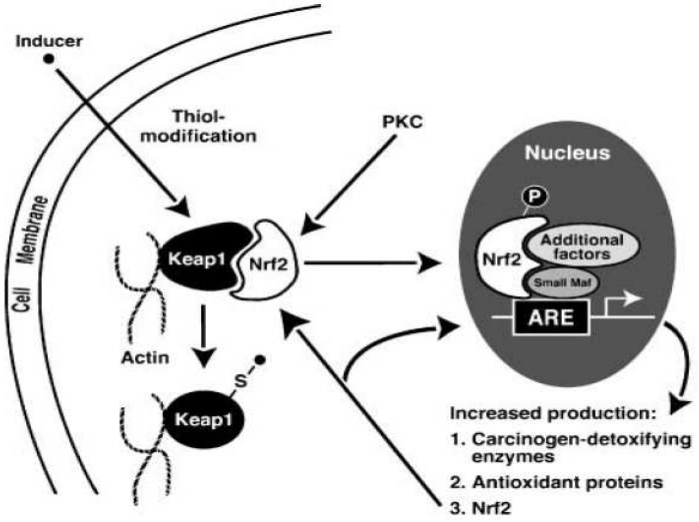


Figure 5. Induction of Nrf2 pathways (Zhang and Gordon, 2004)

4.4.2 Non-toxic storage form of cysteine

Tissue concentrations and plasma levels of cysteine are appreciably lower than those of the vast majority of amino acids (Cooper, 1983). This is probably not only due to the fact that cysteine is readily oxidized into cystine. Because of its thiol group, cysteine is highly reactive. This reactivity is complex and presents a paradox. Indeed, on the one hand, cysteine is an antioxidant that protects cells against otherwise lethal doses of hydrogen peroxide; whereas, on the other, in the presence of transition metals (e.g. Fe and Cu, that frequently have unpaired electrons), cysteine consumes oxygen, generates hydrogen peroxide, and is cytotoxic (Nath and Salahudeen, 1993). GSH represents, therefore, a non-toxic storage form of cysteine (Cooper and Kristal, 1997).

4.4.2.1 Astrocytes provide cysteine to neurons

In the extracellular space, cysteine is readily oxidized to cystine. Cystine can be taken up by astrocytes via the system x_c^- (figure 4), a cystine-glutamate exchange transporter (Cho and Bannai, 1990). Structurally, system x_c^- is composed of a light-chain subunit (xCT), which confers substrate specificity (Sato et al., 1999), and a heavy-chain subunit (4F2) common to the transporter family (Mastroberardino et al., 1998). Immature neurons, cultivated for only 24- to 72-h take up cystine via this transport system (Murphy et al., 1990). This cystine uptake in neurons was shown to come along with an increase in GSH content in neurons cultured for 4 to 5 days, both cystine uptake and GSH levels decreasing thereafter (Sagara et al., 1993). Indeed, although cultured hippocampal neurons are capable of transporting cystine (Allen et al., 2001), neurons in general seem to have a preference for the transport of cysteine (Shanker et al., 2001). Cysteine is transported via a cysteine-glutamate sodium-dependant transporter, the excitatory amino acid transporter (EAAT), with a higher uptake in neurons in comparison to astrocytes (Shanker et al., 2001). Mice lacking EAAC1, that is widely expressed in neurons, show neuronal GSH deficiency (Aoyama et al., 2006). In parallel, it has been demonstrated that the presence of astrocytes strongly increases the content of GSH in neurons (Bolaños et al., 1996). It now seems evident that astrocytes are really important in providing cysteine to neurons (figure 6; Dringen et al., 1999;Sagara et al., 1993). Indeed, GSH is released by astrocytes, cleaved to cysteinylglycine and the cysteine released by the action of dipeptidases can be transported inside neurons before its oxidation (figure 6A; Dringen et al., 2000). Another possibility is that cystine present

in the extracellular space is reduced by GSH released from astrocytes, releasing cysteine that can be taken up by neurons (figure6B; Wang and Cynader, 2000). Finally, homocysteine is a third source of cysteine. Homocysteine can be metabolized via transmethylation reactions, during which it is converted to methionine via the methionine synthase. During oxidative stress, this reaction is decreased and homocysteine is directed towards the transsulfuration pathway (Mosharov et al., 2000;Vitvitsky et al., 2006), where homocysteine is metabolized to cystathionine and subsequently to cysteine (figure 6C). It also seems like GSH is necessary for methionine synthase activity (Muratore et al., 2006).

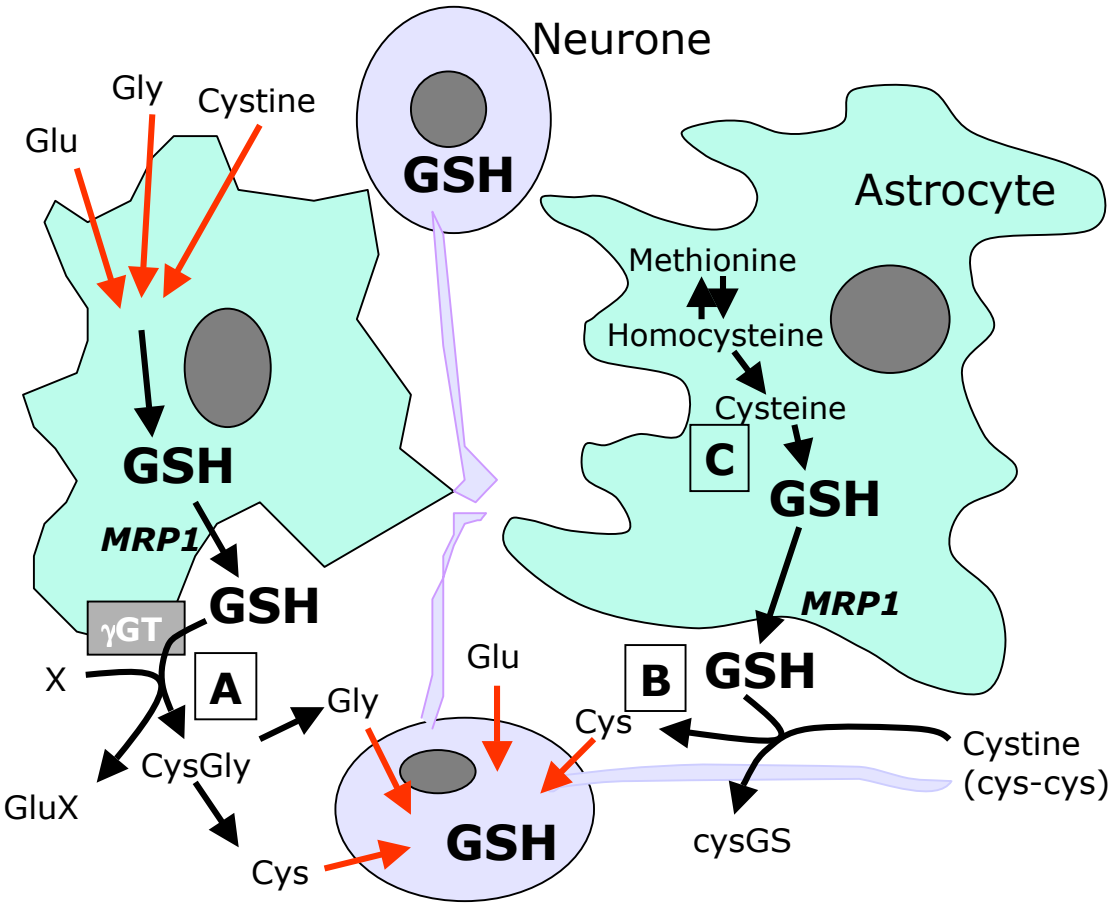


Figure 6. Astrocytes provide neurons with the necessary precursors for the synthesis of GSH.

4.5 Objectives of the present thesis work

Based on impairement of GSH synthesis at the level of the limiting synthesizing enzyme GCL in schizophrenia, the aim of the present thesis work was to find substances

that could lead to an increase in GSH levels in systems with compromised GCL activity. Different strategies can be use to achieve this aim. The first strategy consists in using substances that could bypass GCL and directly increase GSH levels in astrocytes and neurons. The second strategy consists in the induction of GCL and the increase of its activity in the same cell types. Finally, a cysteine donor, the limiting precursor in the synthesis of GSH, was used in a clinical trial with schizophrenia patients.

5. Strategy 1: Bypassing the limiting enzyme GCL

5.1 Introduction

In schizophrenia patients, the deficit in GSH observed is due to a defect at the level of the limiting synthesizing enzyme, GCL (Gysin et al., 2005;Tosic et al., 2006). Bypassing this enzyme would therefore be a considerable possibility to increase GSH levels. Since GSH itself is not transported inside the cell (Meister, 1989), membrane permeable esterified analogs of GSH and its precursor γ-glutamyl-cysteine (γ-glucys) were used.

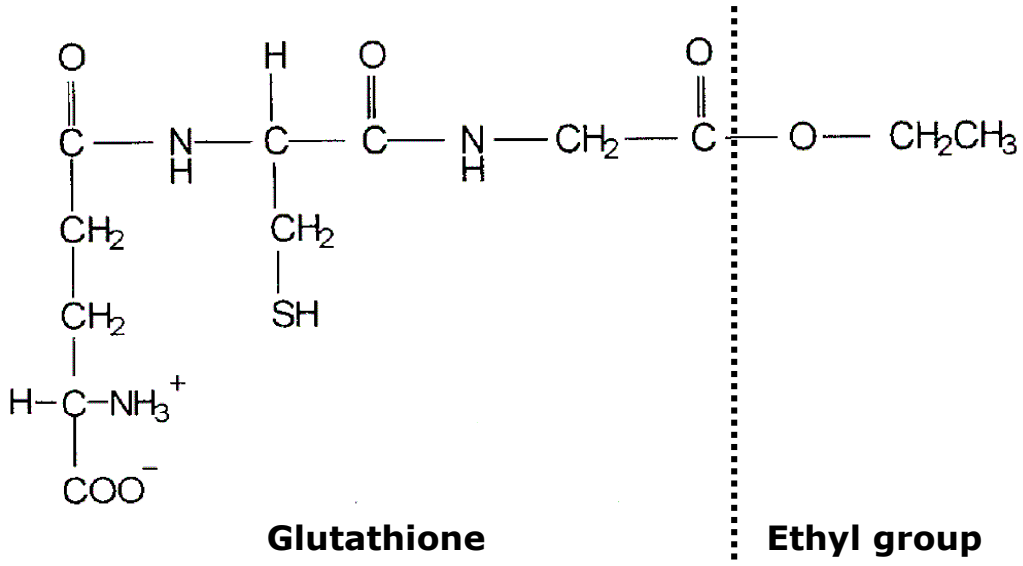


Figure 7. Glutathione Ethyl Ester (GSHEE)

5.1.1 Glutathione Ethyl Ester

Glutathione ethyl ester (GSHEE; figure 7) is the product of the esterification of a molecule of ethanol on the glycine carbon group of GSH. The negative charge on the

glycine is not present anymore, so GSHEE is membrane permeable and transported inside most cells. Inside the cell, GSHEE is converted by esterases into GSH, that can directly be used (figure 8; Anderson et al., 1985). GSH deficiency induced by inhibition of its synthesis by L-buthionine-(S,R)-sulfoximine (BSO), a specific inhibitor of GCL, may be prevented or reversed by administration of GSHEE (Meister, 1991). Indeed, when GSHEE is i.p.-injected (Puri and Meister, 1983) or orally administered (Anderson et al., 1985), GSH levels are increased in liver and kidney of mice pretreated with BSO and protected these organs against acetaminophen toxicity (Puri and Meister, 1983). The same group showed that the ester of GSH could increase GSH levels in cultured human lymphoid cell line and protect these cells from the lethal effects of radiation. They also observed an increase in GSH levels after treatment with GSHEE in cultured skin fibroblasts from patients with GCL deficiency (Wellner et al., 1984). More recently, GSHEE was shown to successfully increase GSH levels in both BSO- and non BSO-treated in mixed cell cultures from rat mesencephalon (Zeevalk et al., 2007), as well as in primary cultured neurons from rat with small interfering RNA-mediated disruption of GCL activity (Diaz-Hernandez et al., 2005).

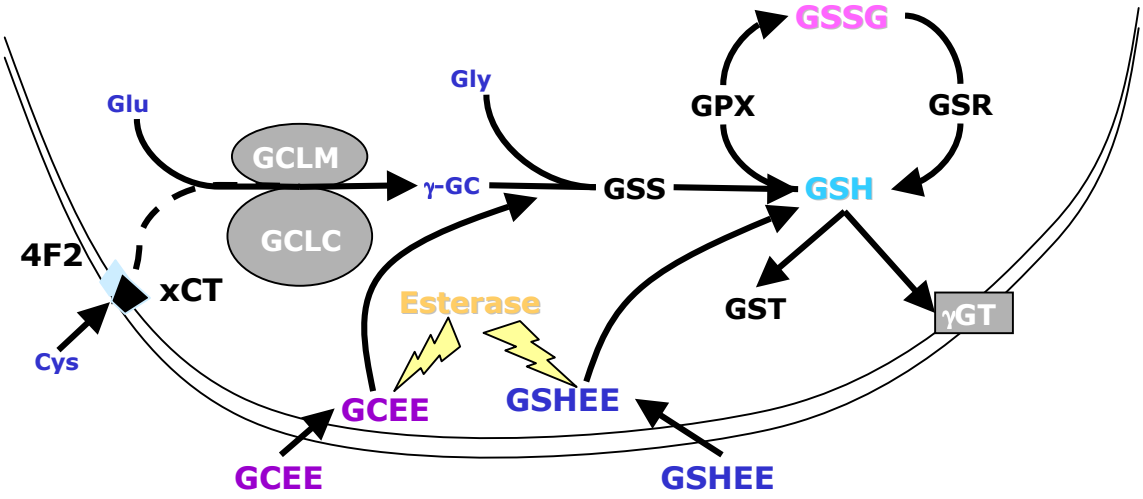


Figure 8. GSHEE and GCEE enter the cell, are de-esterified, and GSH or γ-glucys can readily be used.

5.1.2 γ-Glutamylcysteine Ethyl Ester

γ-Glucys is transported into some cells, such as kidney cells, and used by GSS to form GSH (Anderson and Meister, 1983). Intracerebroventricular administration of γ-glucys

has been shown to increase rat brain GSH levels (Pileblad and Magnusson, 1992). γ-Glucys ethyl ester (GCEE; figure 9) is the product of the esterification of a molecule of ethanol on the cysteine carbon group of γ-glucys. This ester was shown to be transported in more cells than γ-glucys itself (Anderson and Luo, 1998), showing that this ester moiety increases the efficacy of the substance to cross the plasma membrane. Treatment with this esterified GSH precursor in BSO-treated neurons could increase GSH levels back to control levels and could protect non-BSO treated neurons against β-amiloid-induced oxidative stress, a model for Alzheimer's disease (Boyd-Kimball et al., 2005). The capacity of GCEE in increasing GSH levels after BSO treatment was also demonstrated in N27 cells, which are derived from embryonic rat mesencephalic neurons (Chinta et al., 2006).

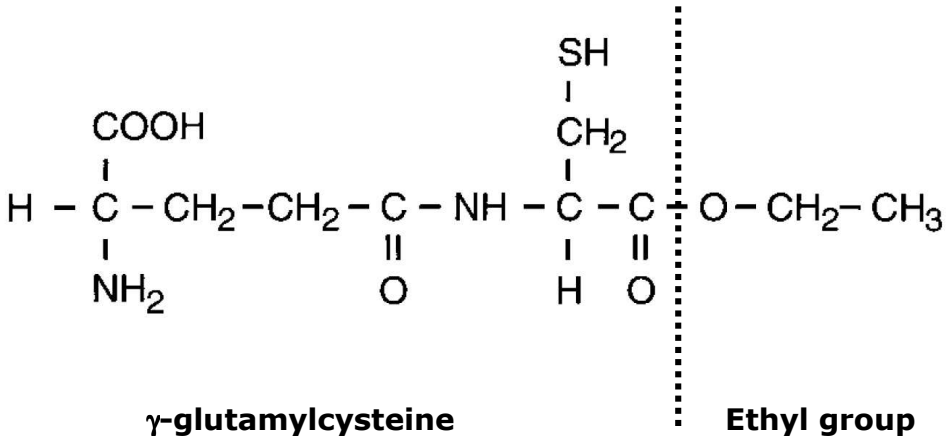


Figure 9. γ-glutamylcysteine Ethyl Ester (GCEE)

In the present study, we evaluated the efficacy of GSHEE and GCEE in increasing GSH levels in cultured neurons and astrocytes from mice, when GCL was inhibited by BSO. In addition, we investigated whether such compounds could prevent the effect of a GSH deficit on NMDA responses.

5.1.3 Effect of GSH deficit and replenishment on NMDA response

We recently observe that neurons with a deficit in GSH induced by BSO, show an altered modulation of NMDA-mediated calcium response (NMDA responses) by dopamine (Steullet et al., submitted). In control neurons, dopamine enhanced NMDA responses likely via D1 receptors. In BSO-treated neurons, dopamine decreased NMDA responses via D2 receptors. This difference in dopamine modulation of NMDA

responses was mostly explained by a differential modulation of L-type calcium channels. The redox-sensitive ryanodine receptors (RyRs), which were enhanced in BSO-treated neurons, played an essential role in altering dopamine signaling in neurons with a GSH deficit. Data suggest that enhancement of the function of RyRs in neurons with low GSH levels favors D2-type receptor-mediated and calcium-dependent pathways, causing a change in dopamine modulation of L-type calcium channels and ultimately in dopamine modulation of NMDA responses. We therefore investigated whether increasing GSH levels with GSHEE in BSO-treated neurons would reverse the effects observed on the dopamine modulation of NMDA responses.

5.2 Material and methods

5.2.1 Primary cultures of cortical neurons

Cultures of cortical neurons were prepared from E16-17 OF1 mice (S/IOPS OF1 from Charles River Laboratories, L'Arbresle, France) in accordance with the authorization issued by the "Office Vétérinaire du Canton de Vaud". The pregnant female mouse was decapitated, then brains of embryos were removed and the cortices were isolated 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, 1 mM sodium pyruvate, pH = 7.4) containing penicillin (100 u/ml) and streptomycin (100µg/ml). Cortical cells were mechanically dissociated with a Pasteur pipette, centrifuged and resuspended in Neurobasal medium (Invitrogen) supplemented with 2% B27 (Invitrogen), 500 µM glutamine and 30µM glutamate. Neurons were plated on poly-L-ornithine-coated culture dishes at a density of 800 cells/mm^2 or on poly-L-ornithine-coated glass coverslips at a density of 600 cells/mm^2. Cells were maintained at 37°C and in a humidified 5% CO_2 atmosphere.

5.2.2 Primary cultures of cortical astrocytes

Cortices from P1-3 OF1 mice (S/IOPS OF1 from Charles River Laboratories, L'Arbresle, France) were dissected in DMEM medium (Invitrogen, Carlsbad, CA, USA) supplement with 10% fetal bovine serum (Invitrogen) and containing penicillin (100 u/ml) and streptomycin (100µg/ml). Cortical cells were mechanically dissociated through needles with decreasing diameters, centrifuged at 500 rpm for 5 min 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.

5.2.3 Treatments

Four to six days-old neurons and 14-15 days-old astrocytes were treated as shown on figure 10. GSH deficit was induced with L-buthionine-(S,R)-sulfoximine (BSO), a specific inhibitor of GCL. A dose-response curve showing the effect of BSO on GSH levels was established for the two cell types. Both 100µM-treatment BSO in neurons and 10µM-treatment BSO in astrocytes led to a decrease in GSH levels of about 70%. Cells were incubated for 24 hours in Neurobasal medium containing 100µM BSO for neurons and 10µM BSO for astrocytes, supplemented with 500µM glutamine and 2% special B27 without antioxidants (Invitrogen, Carlsbad, CA, USA). Controls were incubated in the same medium, but without BSO. Seven hour after the beginning of BSO incubation, either GSHEE or GCEE was added, or not, to the medium for 24h. GSH levels and LDH activity were then assessed.

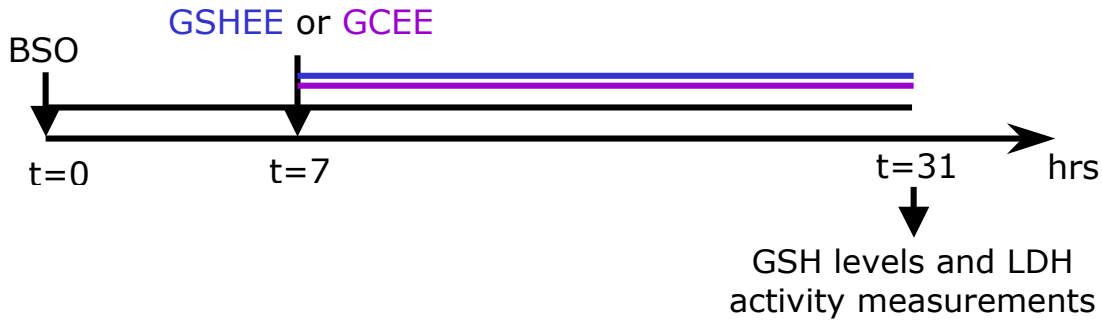


Figure 10. Experimental design

5.2.4 Total GSH measurements

Quantification of total GSH content in cells was performed with an assay based on the Tietze method (Tietze, 1969). Cells were washed twice with ice-cold PBS (150mM NaCl, 3mM KCl, 1.5mM KH_2PO_4 and 7.9mM Na_2HPO_4), and scrapped out of the dish with 100 µl ice-cold PBS containing 1-2.5mM EDTA. After sonication, an aliquot was reserved for subsequent analysis of protein content using the Advanced Protein Assay (Cytoskeleton, Denver, CO, USA). Proteins in the remaining cell solution were precipitated with 5-Sulfosalicylic acid (SSA) and precipitate was removed from solution

by centrifugation. Triethanolamine (TEA) was added to the supernatant to bring pH >7. In a well of a 96-well plate, 100 µl of supernatant diluted in PBS as much as needed, was mixed with 100 µl of freshly made reaction solution [PBS with 2.5mM EDTA, 0.3mM DTNB, 0.3mM NADPH and 1 U/ml GSH reductase]. The absorbance at 405 nm was measured with a plate reader 20 sec after mixing and then at 15 sec intervals for 1 min. The rate of increase in absorbance, which measures the cyclic reduction of DTNB by GSH, was proportional to the total GSH. Measurements were done in duplicates. Concentration of GSH in sample solution was determined by comparison with a series of freshly-made GSH standard solutions. GSH content in cells are expressed in nmole GSH/mg proteins. The possible presence of GSHEE in samples from GSHEE-treated neurons did not interfere with the measurements of GSH as verified using standard solutions of GSHEE alone or with GSH.

5.2.5 Assessment of cell viability

Cell viability was assessed by measuring the activity of lactate dehydrogenase (LDH) in the culture medium (adapted from Dringen et al., 1998). When cells are dying, cell membranes become permeable and cells release their content, including LDH, in the medium. An aliquot of culture medium was collected and kept at – 80°C. For measurement of LDH activity, 50 µl of the collected medium was mixed with 100 µl of 0.1M potassium phosphate buffer (pH 7.4) and 150 µl of a freshly-made reaction solution [6.4mM sodium pyruvate and 0.8mM NADH in 0.1 M potassium phosphate buffer]. The decrease in absorbance at 340 nm, recorded every minute for 10 min using a plate reader, was proportional to LDH activity. Measurements were done in duplicates. LDH activity in the medium was scaled with the activity of a series of LDH standard solutions. The amount of LDH in the medium was then reported to the amount of proteins measured in the cells of the corresponding dish.

5.2.6 Calcium experiments

Changes in intracellular calcium concentrations were measured at room temperature with the calcium indicator dye, Fluo-4AM (Molecular probes, Invitrogen, Carlsbad, CA, USA; Thomas et al., 2000). Fluo-4 was dissolved to 5mM in DMSO/20% pluronic F-127 (Molecular probes, Invitrogen, Carlsbad, CA, USA), and stored at –20°C until use. A poly-L-ornithine-coated coverslip with attached control or treated neurons was

washed twice with HEPES buffer (140mM NaCl, 5mM KCl, 20mM glucose, 2mM $CaCl_2$, 10mM HEPES, pH=7.35), incubated in the dark for 30 min in HEPES buffer containing 5 µM Fluo-4AM, and kept for 30 min in HEPES buffer to allow de-esterification of the dye. The coverslip was then washed with HEPES buffer, mounted on a recording chamber, and placed on an epifluorescence microscope (Nikon Eclipse TE300, Nikon Inc., Japan). HEPES buffer flowed continuously at ~1 ml/min through the recording chamber. Images were collected every 2 s with a CCD camera (Princeton Instruments, Trenton, NJ, USA) coupled to an acquisition system (Metamorph, Universal Imaging Corporation, West Chester, PA, USA). The excitation light was provided by a 100 W Xenon lamp. The excitation and emission filters were 465-495 nm and 515-555 nm, respectively. A fast step perfusion system (Warner Instruments Corporation, Hamden, CT, USA) was used to present stimuli over the neurons of interest. Images were analyzed off-line with the software Metamorph (Universal Imaging Corporation, West Chester, PA, USA). The fluorescence of each neuron was corrected by subtraction of the background fluorescence that was determined in areas with no cells. Calcium-sensitive fluorimetric changes upon stimulation were normalized with the baseline fluorescence (F0) measured before stimulation, and were expressed as 100*(delta F)/F0, where delta F was the fluorescence change induced by a stimulation. Responses were quantified at their peak and were compared across treatments using sets of neurons from sister culture dishes of a same cultures. NMDA-mediated calcium responses were typically induced by a mixture of 5µM NMDA and 5µM D-serine. Dopamine modulation of calcium responses was investigated by superfusing freshly-made dopamine for 1 min before and during the NMDA stimulation.

5.2.7 Statistical analysis

The effect of treatments (presence or absence of BSO and GSHEE) on GSH content or cell viability (dependent variables) was analyzed by one-way ANOVA followed by planned pair-wise comparisons between treated groups. Statistical significance was corrected for multiple comparisons using the sequential Bonferroni test with the Dunn-Šidák method (Sokal and Rohlf, 1997). Significant probability level was set to $p \leq 0.05$

Because NMDA-mediated calcium responses usually did not follow normal distributions according to the Shapiro-Wilk test, non-parametric statistic was used. First, the effects of BSO and GSHEE treatment (presence or absence of treatment) and

dopamine (absence vs. presence) on calcium responses (dependent variable) were analysed using a two-way ANOVA for ranked data [Scheirer-Ray-Hare extension of the Kruskall-Wallis test (Sokal and Rohlf, 1997)]. Planned pair-wise comparisons of calcium responses between groups were then performed with Kruskall-Wallis test and statistical significance was corrected for multiple comparisons as previously described. Significant probability level was set to $p \leq 0.05$.

5.3 Results

5.3.1 Effect of GSHEE on cultured astrocytes and neurons

In both neurons and astrocytes, GSH deficit was induced by 24-h treatment with L-buthionine-(S,R)-sulfoximine (BSO), an inhibitor of GCL. In neurons (figure 11A), a significant 70% decrease in GSH levels ($p < 0.001$) was observed after a treatment with 100µM BSO. A 24-h treatment with 1mM GSHEE significantly increased GSH levels in both control ($p < 0.001$) and BSO-treated neurons ($p < 0.001$). In BSO-treated neurons, GSHEE increased GSH levels to levels similar to those measured in control neurons. Cell viability was affected neither by BSO nor by GSHEE treatment, as assessed by measuring the activity of LDH from culture media (figure 11B).

In astrocytes, a significant 73% decrease in GSH levels ($p < 0.001$) was observed after a treatment with 10µM BSO (figure 12A). Compared to neurons, astrocytes appear to be more sensitive to BSO. Indeed, the same percentage of decrease in GSH levels was obtained with 10 times less BSO in astrocytes compared to neurons. Higher concentrations of GSHEE are necessary to increase GSH levels in astrocytes compared to neurons. In BSO-treated astrocytes, 3mM GSHEE fully replenished GSH contents to levels similar to those observed in control astrocytes ($p < 0.001$). 1mM or 2mM GSHEE only partially increased GSH levels in a dose-dependant manner. By contrast with neurons, 1mM, 2mM or 3mM GSHEE, did not lead to any significant increase in GSH levels in non-BSO treated astrocytes. Cell viability was affected neither by BSO nor by GSHEE treatment, as assessed by measuring the activity of LDH from culture media (figure 12B).

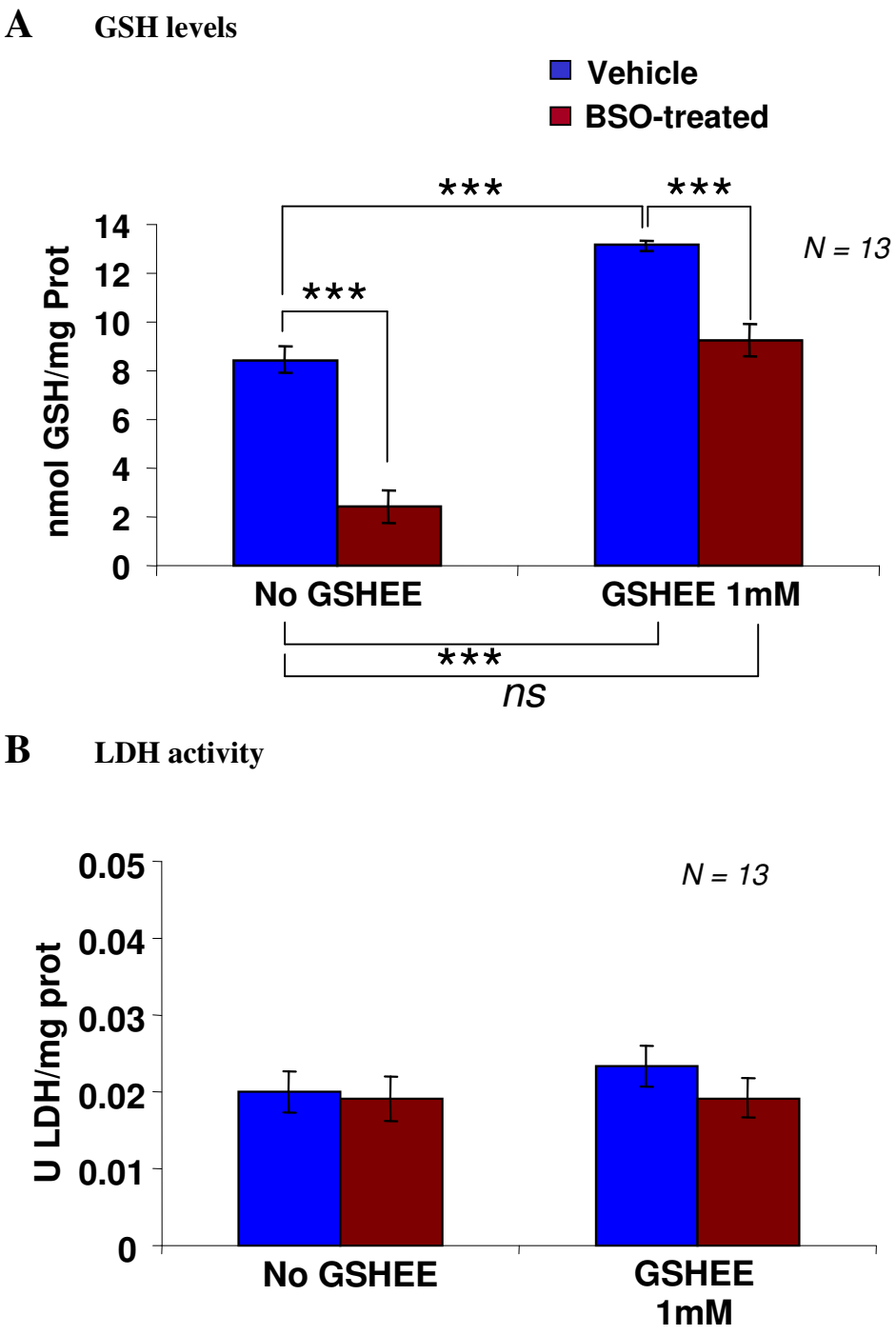


Figure 11. **GSHEE in Neurons.** Effects of BSO and GSHEE treatments on total GSH levels (A) and LDH activity (B) in neurons. Data are presented by the mean ± SEM. ***, p<0.001.

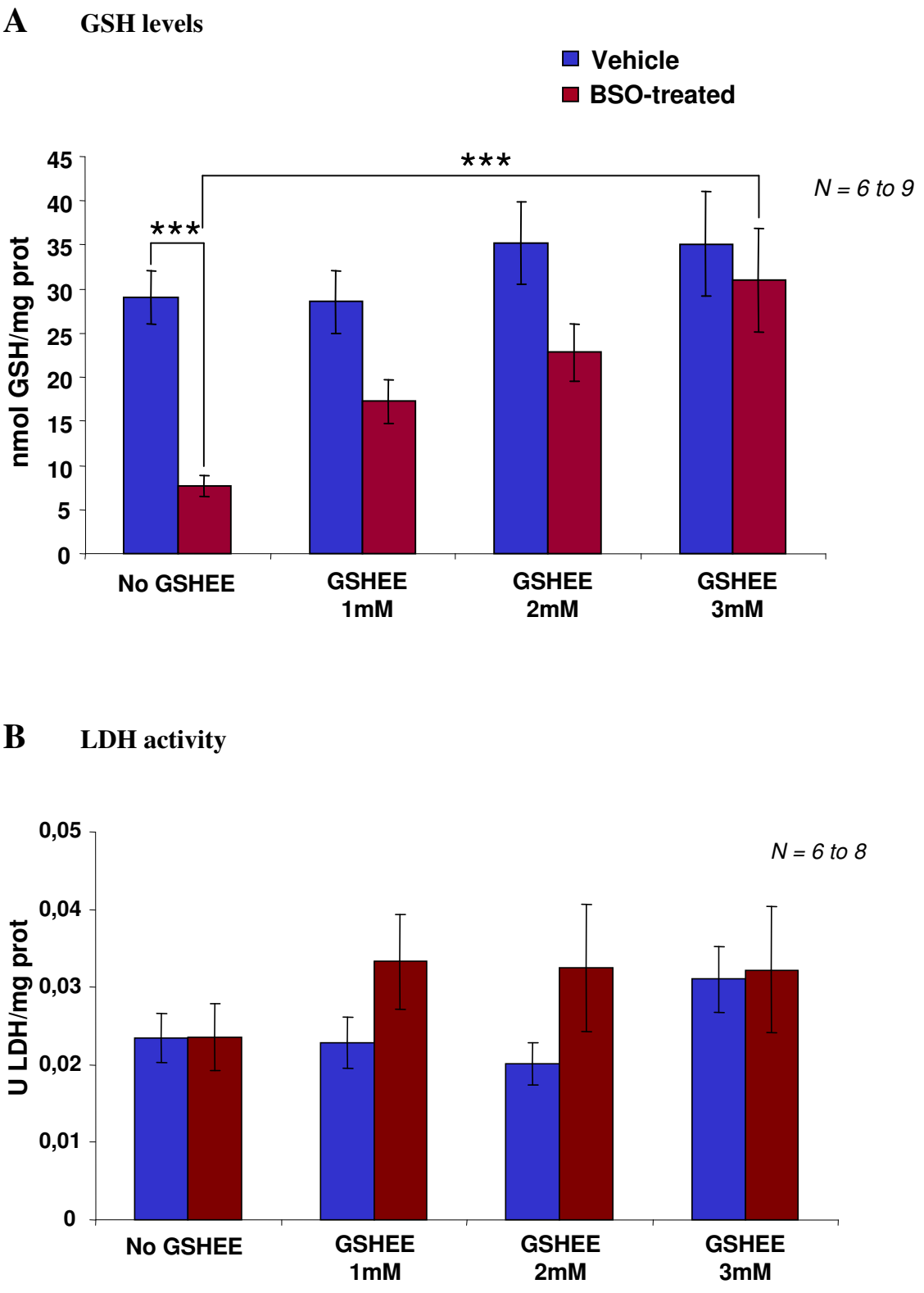


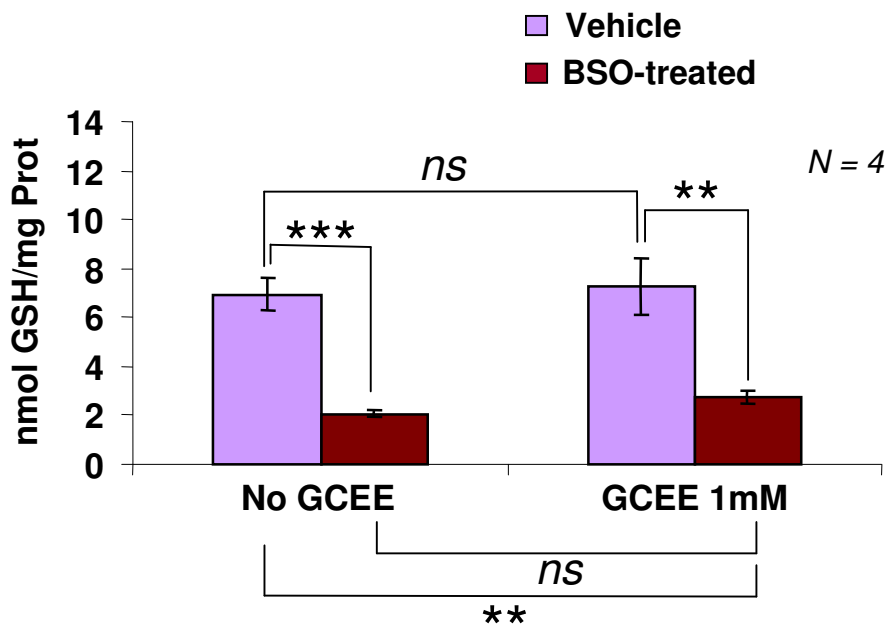
Figure 12. **GSHEE in astrocytes**. Effects of BSO treatments and various concentrations of GSHEE on total GSH levels (A) and LDH activity (B) in astrocytes. Data are presented by the mean ± SEM. ***, $p<0.001$.

6.3.1 Effect of GCEE on cultured astrocytes and neurons

By contrast with GSHEE, 24-h treatment with 1mM GCEE did not increase significantly GSH levels in neither control nor BSO-treated neurons (figure 13A). As assessed by measuring the activity of LDH from culture media, cell viability was affected neither by BSO nor by GCEE treatment (figure 13B).

However, 3mM GCEE was efficient in raising GSH levels in BSO-treated astrocytes to levels similar to those observed in controls ($p < 0.001$; figure 14A). 1mM or 2mM GCEE only partially increased GSH levels in a dose-dependant manner. Treating control astrocytes with 1mM, 2mM or 3mM GCEE, did not lead to any significant increase in GSH levels. As assessed by the measurement of the activity of LDH from culture media, cell viability was not affected neither by BSO nor by GCEE treatment (figure 14B).

A **GSH levels**



B **GCL activity**

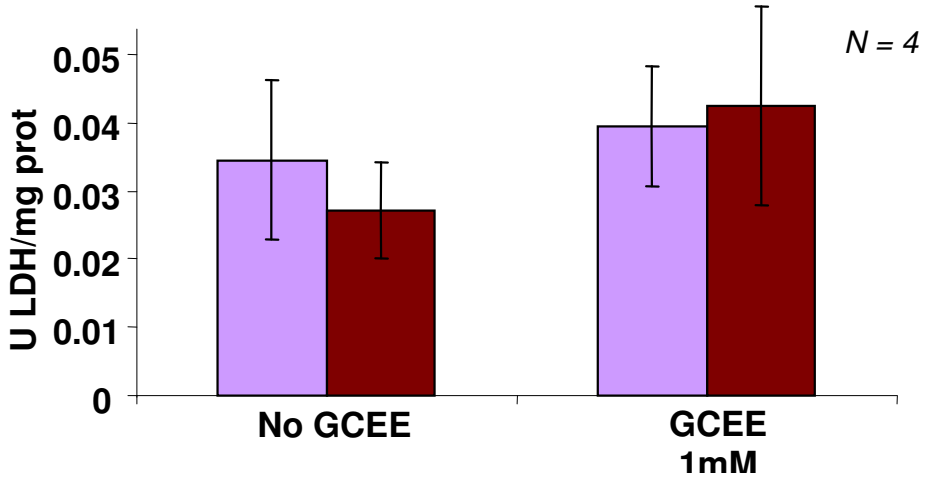


Figure 13. **GCEE in neurons.** Effects of BSO and GCEE treatments on total GSH levels (A) and LDH activity (B) in neurons. Data are presented by the mean ± SEM. **, p<0.01; ***, p<0.001.

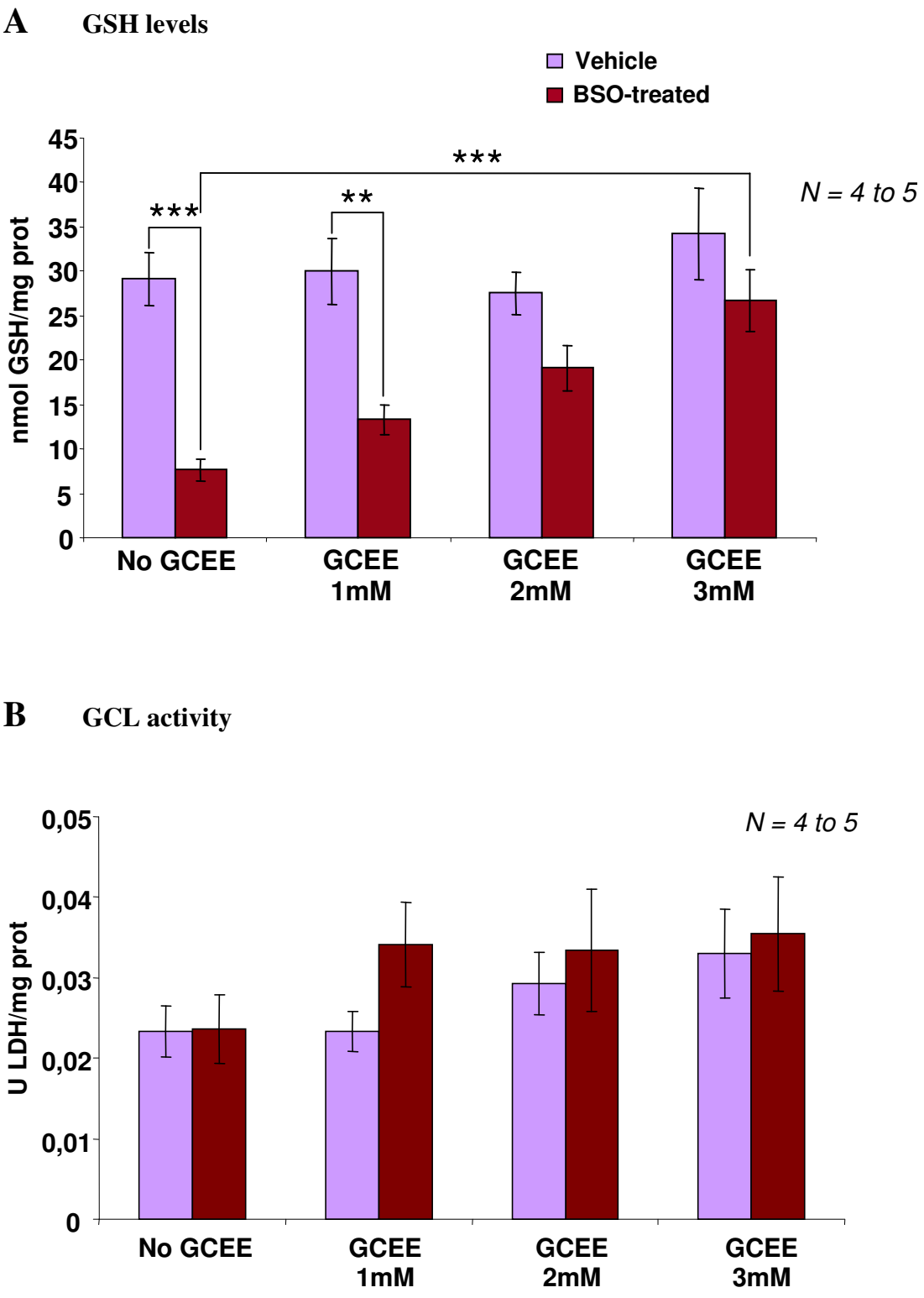


Figure 14. **GCEE in astrocytes.** Effects of BSO treatments and various concentrations of GCEE on total GSH levels (A) and LDH activity (B) in Astrocytes. Data are presented by the mean ± SEM. **, p<0.01; ***, p<0.001.

5.3.3 Effect of GSH replenishment on NMDA response

The effect of a deficit in GSH in neurons on NMDA-mediated calcium responses (NMDA response) was assessed (figure 15). There was no difference between NMDA responses in control and BSO-treated neurons. We then investigated whether a GSH deficit affected the modulation of NMDA responses by dopamine. Superfusion of dopamine (1 min, 1µM) changed the magnitude of NMDA responses; this modulation of NMDA responses was different in BSO-treated and in control neurons. There were significant effects of BSO treatment ($p < 0.001$) and a significant interaction between BSO treatment and dopamine ($p < 0.001$) as revealed by a Scheirer-Ray-Hare two-way ANOVA performed on NMDA responses. When GSH deficit was induced, the presence of dopamine, significantly decreased NMDA responses compared to NMDA responses measured in the absence of dopamine in BSO-treated neurons ($p < 0.01$) and in the presence of dopamine in control neurons ($p < 0.001$).

Treatment with 1mM GSHEE significantly increased GSH contents in BSO-treated neurons to levels similar to those measured in control neurons (figure 11A). We therefore investigated whether restoring GSH levels in BSO-treated neurons would abolish the dopamine-induced decrease of NMDA responses. When GSH levels were increased with GSHEE in BSO-treated neurons, dopamine no longer decreased NMDA responses (figure 15). Indeed, GSHEE significantly increased NMDA responses of BSO-treated neurons under dopamine superfusion compared to BSO-treated neurons in the absence of GSHEE ($p < 0.01$). These results demonstrate that a depletion of intracellular GSH was responsible for the dopamine-induced decrease of NMDA responses observed in BSO-treated neurons. It also showed that GSHEE can remediate to the deleterious effect of a blockade of GSH synthesis on NMDA-dependent calcium responses.

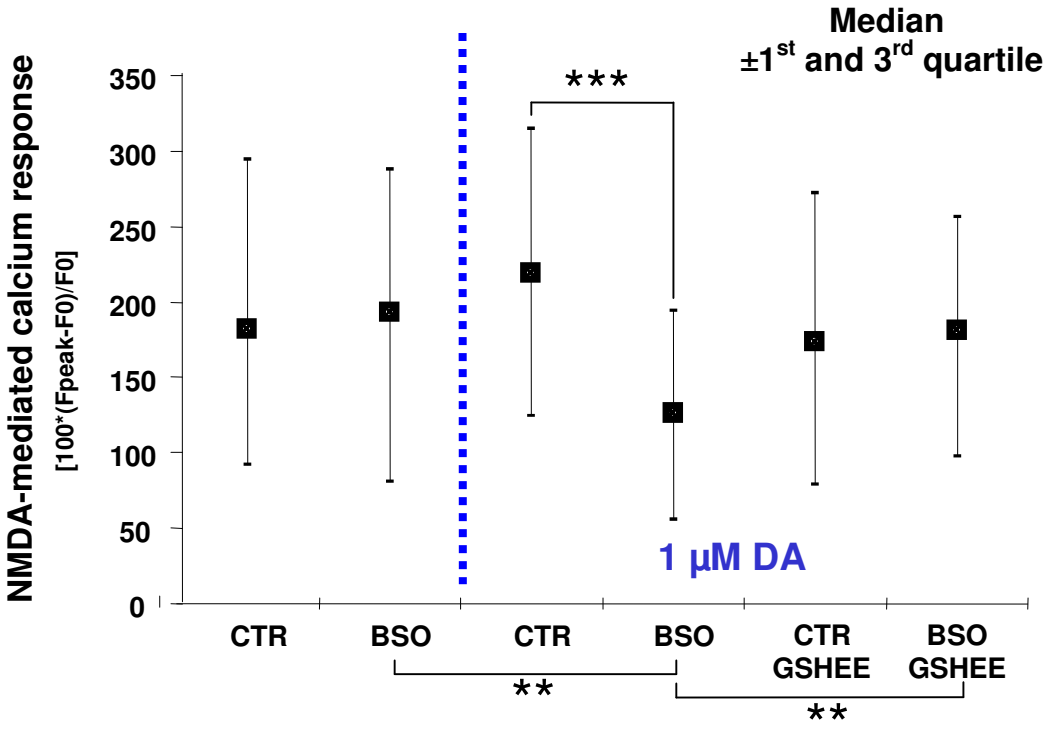


Figure 15. Effects of BSO-induced GSH deficit on dopamine modulation of NMDA responses (n = 91-115) and effects of re-establishment of GSH levels with GSHEE on that modulation (n = 120-122). Data are presented by the median and quartiles. **, p<0.01; ***, p<0.001.

6. Strategy 2: Boosting the rate-limiting enzyme GCL

On section 5, we saw that is it possible to increase GSH levels using substances that bypass GCL, the enzyme that is responsible for the deficit in GSH observed in schizophrenia patients (Gysin et al., 2005;Tosic et al., 2006). *GCLM* and *GCLC* being two phase II genes, we hypothesized that it would be possible to increase their expression using substances known to induce these genes. To test this strategy, curcumin (polyphenol), quercetin (flavonoid), *tert*-butylhydroquinone (quinone) and FK506 (immunosuppressant) were used (table 1). GSH acts as an antioxidant via the enzyme GST and, via the redox cycle, acts as a free radical scavenger (Pias and Aw, 2002). The effects of the substances on the redox status inside the cells, was assessed via the measurements of both GSH and GSSG levels. The actvity of GST was measured in order to observe a potentatial activation of the transferase pathway, by which GSH plays its antioxidant role. Of course, since the main idea is to boost GCL, the activity of

this enzyme was measured, as well as the cell viability. Furthermore, in an attempt to better understand the mechanisms by which they could produce their effects, we measured the expression of genes implicated in GSH metabolism: the transcription factors Nrf2, the two subunits of the cysteine-glutamate exchanger, xCT and 4F2, the two subunits of GCL, GCLM and GCLC, as well as GSS and GPx. All these measurements were done in both astrocytes and neurons. Finally, because of the evidence suggesting a link between GCLM and schizophrenia, we assessed the necessity of a functional GCLM in the action of the substances used. This was done using astrocytes from GCLM KO mice.

Substance	Cell type	Mechanisms	Reference
Curcumin	Human erythroleukemia cell line (K562)	Increase in GCL activity	Singhal *et al*, 1999
	Rat type 1 astrocytes cell line (DI TNC1)	Increase in Nrf2 protein levels and translocation of Nrf2 to the nucleus	Scapagnini *et al*, 2002
	Human bronchial epithelial cell line (HBE1)	Increase in mRNA and protein levels of GCLC and GCLM	Dickinson *et al*, 2003
	Human myelomonocytes (U937)	Not investigated	Strasser *et al*, 2005
	Human alveolar epithelial cell line (A 549)	Increase in mRNA levels of GCLC; GCLM not investigated	Biswas *et al*, 2005
Quercetin	Human breast cancer cell line (MCF7)	Not investigated	Rodgers and Grant, 1998
	Monkey kidney derived cell line (COS-1)	Increase in mRNA of GCLC and GCLM	Myhrstad *et al*, 2002
	Human hepatoma cell line (HepG2)	Increase in GCL activity	Scharf *et al*, 2003
	Human hepatoma cell line (HepG2)	Not investigated	Alia *et al*, 2005
tBHQ	Human hepatoma cell line (HepG2)	Increase in mRNA of GCLC and GCLM	Galloway *et al*, 1997
	Primary cortical astrocytes from mouse	Not investigated	Eftekharpour *et al*, 2000
	Primary cortical astrocytes from mouse	Translocation of Nrf2 to the nucleus leading to ARE activation	Lee *et al*, 2003
	Human bronchial epithelial cell line (HBE1)	Increase in mRNA of GCLC and GCLM and protein levels of GCLC only	Krzywanski *et al*,2004
	Primary cortical astrocytes from rat	Upregulation of Nrf2 pathway	Sun *et al*, 2005
	Primary astrocytes from spinal cord of rat	Induction of phase II enzymes	Vargas *et al*, 2006
FK506	Neuroblastoma cell line (Neuro 2A)	Not investigated	Tanaka *et al*, 2000
	Neurobastoma-glioma hybrid cell line (NG108-15)	Not investigated	Tanaka *et al*, 2001
	Glioma cell line (C6)	Not investigated	Tanaka *et al*, 2002
	Glioma cell line (U251)	Not investigated	Tanaka *et al*, 2004
	Primary cortical astrocytes from rat	Not investigated	Gabryel *et al*, 2005

Table 1. Cell types in which curcumin, quercetin, tBHQ and FK506 could increase GSH levels, and proposed mechanisms underlying this increase.

6.1 Introduction

6.1.1 Curcumin

Diferuloylmethane [1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione], commonly named curcumin (figure 16), is a polyphenol extracted from turmeric, the powdered rhizome of the plant, *Curcuma longa*, which is part of the Zingiberaceae family that also include ginger. This yellow-colored spice is widely used by Asians in the preparation of curry. Curcumin has been the subject of hundreds of papers over the past three decades, studying its antioxidant, anti-inflammatory, cancer chemopreventive and potentially chemotherapeutic properties.

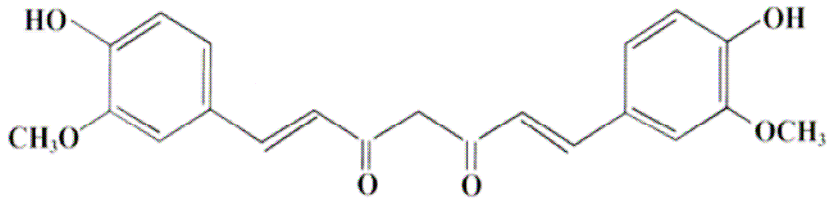


Figure 16. Curcumin

Curcumin was shown to increase GSH in different cell lines, such as cultured human erythroleukemia (K562; Singhal et al., 1999), type 1 astrocytes from rat (DI TNC1; Scapagnini et al., 2002), human bronchial epithelial (HBE1; Dickinson et al., 2003), human myelomonocytes (U937; Strasser et al., 2005) and human alveolar epithelial (A549; Biswas et al., 2005). This increase in GSH levels could be due to an increase of *de novo* GSH synthesis following the increased activity of GCL due to an induction of its expression by Nrf2. Indeed, curcumin was shown to increase Nrf2 protein levels in nuclear extracts from rat type 1 astrocytes (DI TNC1) and Nrf2 translocation to the nucleus was accompanied by an upregulation of some phase II enzymes in these cells (Scapagnini et al., 2006). In human lung epithelial cell lines, an increase in the content of *GCLC* (Biswas et al., 2005) and *GCLM* mRNA, as well as in the content of both GCL subunit proteins was observed after a treatment with curcumin (Dickinson et al., 2003). Finally, curcumin induced 1.6 fold increase in GCL activity in K-562 cells leading to a 1.2 fold increase in GSH levels (Singhal et al., 1999).

6.1.2 Quercetin

Flavonoids are polyphenolic compounds that include more than 6 000 naturally occurring substances that can be found in every parts of a plant. They are responsible for the color of many fruits, vegetables and flowers. Thus, we can find flavonoids in tea, fruits and vegetables as well as in red wine. Flavonoids possess a skeleton made of 15 atoms of carbon arrange in two aromatic rings linked by a linear chain of three carbons. Seven major subgroups can be found in most major plants: chalcones, flavones, flavonols, flavandiols, anthocyanins condensed tannins and aurones (Winkel-Shirley, 2001). Subsequent steps, in particular glycosylation and acylation, bring flavonoids to their definitive structure that can be found *in vivo*. Like GSH, flavonoids possess antioxidant properties, as well as the capacity to eliminate free radicals. These attributes made flavonoids responsible for the well know phenomenon named “French paradox”. In spite of a high risk factor to develop conditions like cholesterol, diabetes, hypertension due to a high ingestion of saturated fatty acid, French peoples show the lowest mortality from coronary heart disease among industrialized occidental countries (Renaud and de Lorgeril, 1992). It is possible that this paradox could be explained by the regular red wine consumption in France (Renaud and Gueguen, 1998).

During an oxidative stress, GSH levels decrease, ROS levels increase and that is accompanied by a massive entrance of calcium Ca^{2+} in the cell, followed by cell apoptosis (Tan et al., 1998). Therefore, substances that protect cells against oxidative stress can act at various levels, either by increasing GSH levels, neutralizing ROS or preventing the massive Ca^{2+} entrance. Flavonoids were classified into three categories according to the protection mechanism they use (Ishige et al., 2001). Thus, during a glutamate-induced toxicity in neurons, “flavonoles” type maintain low Ca^{2+} levels in spite of the presence of ROS and the decrease in GSH levels. “Galanguines” neutralize ROS and at last, “quercetines”, in addition to the fact that they neutralize ROS, also lead to an increase in GSH levels. We were therefore interested in the main representative of that very last type of flavonoids, the quercetin itself (figure 17). Quercetin can be found in onions, apples, green or black tea and, in lowest amount, in green leaves vegetables and beans. In addition to its antioxidant properties, quercetin is known for its antihistaminic, anticancer, anti-inflammatory, antiviral and antibacterial properties.

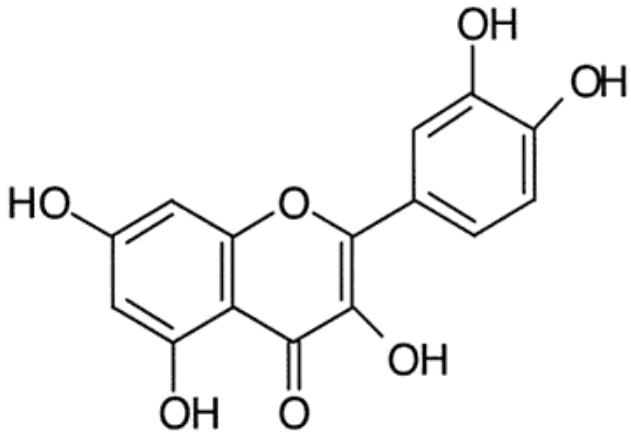


Figure 17. Quercetin

Quercetin induced an increase of GSH levels in human breast cancer cells (MCF7; Rodgers and Grant, 1998), monkey kidney derived cells (COS-1; Myhrstad et al., 2002), human hepatoma cell line (HepG2; Alia et al., 2005;Scharf et al., 2003). The proposed mechanism for the increase in GSH levels by quercetin is the upregulation of the enzyme GCL linked, via the activation of Nrf2 and induction of phase II genes (figure 5). Indeed, it was shown that quercetin is able to induce the ARE-dependent gene, NAD(P)H:quinone oxidoreductase (NQO1; Valerio et al., 2001). More specific to our purpose, the increase in GSH levels in COS-1 cells was accompanied by an increase in *GCLC* and *GCLM* mRNA levels (Myhrstad et al., 2002).

6.1.3 *tert*-Butylhydroquinone

tert-Butylhydroquinone (tBHQ) is a xenobiotic, meaning that this substance is not endogenous. It is a synthetic phenolic antioxidant used as a model inducing agent for ARE-driven transcription (Nguyen et al., 2003). tBHQ is the metabolite of butylated hydroxyanisole, both substances being used as food preservative for their antioxidant properties.

tBHQ increased GSH levels in HBE1 (Krzywanski et al., 2004) and HEPG2 (Galloway et al., 1997) and this was preceded by an increase in both *GCLC* and *GCLM* mRNA, but only GCLC protein levels were increased (Krzywanski et al., 2004). It was also shown to increase GSH levels in astrocytes following the activation of the transcription factor Nrf2 (Lee et al., 2003;Sun et al., 2005;Vargas et al., 2006). In another study, tBHQ also

increased GSH levels in mice cortical astrocytes, but not in neurons (Eftekharpour et al., 2000), suggesting that phase II enzymes can be induced in astrocytes, but with considerably lower levels in neurons (Ahlgren-Beckendorf et al., 1999;Eftekharpour et al., 2000;Shih et al., 2003).

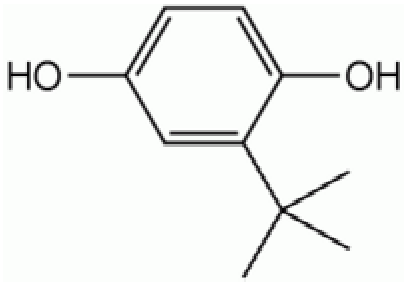


Figure 18. *tert*-Butylhydroquinone

6.1.4 FK506

FK506, also named tacrolimus, is an immunosuppressant widely used for organ transplants. This substance is isolated from the microscopic fungus *Streptomyces tsukubaensis* (Vinet and Busque, 1997). The majority of research on immunophilins, the receptors for FK506, has focused on cells of the immune system, but the finding that the immunophilins are much more abundant in the nervous system led to researches that revealed important roles for the immunophilins in multiple areas of neural function. These include regulation of nitric oxide (NO) neurotoxicity, neurotransmitter release, intracellular Ca2+ flux via the ryanodine and the inositol-(1,4,5)-trisphosphate (IP3) receptors, as well as neurotrophic influences (Snyder et al., 1998).

GSH levels were shown to be increased after FK506 treatment in neuroblastoma cell line (Neuro 2A; Tanaka et al., 2000), in neuroblastoma-glioma hybrid cell line (NG108-15; Tanaka et al., 2001), and in glioma cell lines (C6, U251; Tanaka et al., 2002;Tanaka et al., 2004). However, the mechanisms by which this immunosuppressant can increase GSH levels are unclear. The same group has conducted an *in vivo* study during which FK506 was i.p.-injected to mice and they observed an increase in GSH levels in striatum, as well as an increase in gene expression of GCL. However, no distinction was done between the whole GCL and its subunits, regarding the mRNA quantification, which it quite intriguing. No change in mRNA levels of GPx or GST was observed, leading one to doubt about the phase II enzyme-induction activity of FK506.

Furthermore, in astrocytes depleted in GSH by BSO, another group has observed an increase in GSH levels after FK506 treatment (Gabryel et al., 2005). BSO being an inhibitor of GCL, the action of FK506 is most probably not through the activation of this enzyme. It is proposed that the antioxidant properties of FK506 themselves prevent the decrease in GSH levels (Nishinaka et al., 1993).

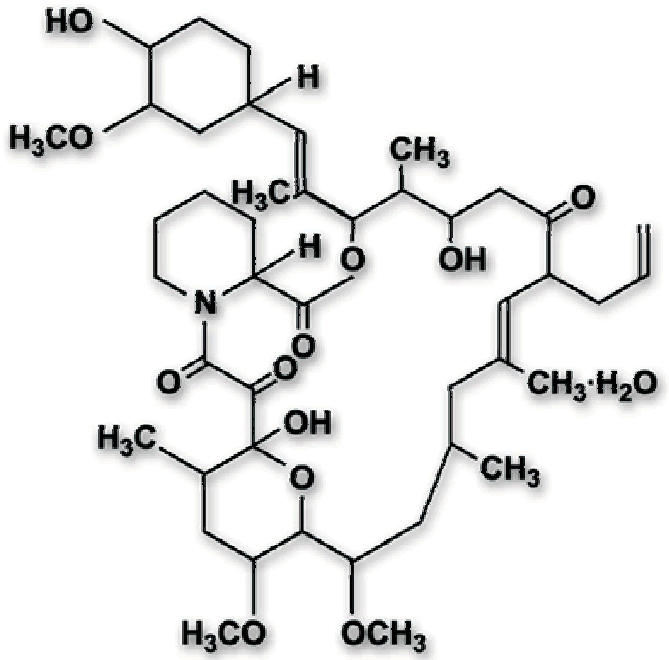


Figure 19. FK506

6.2 Materials and methods

6.2.1 Primary cultures of cortical neurons

Cultures of cortical neurons were prepared from E16-17 OF1 mice (S/IOPS OF1 from Charles River Laboratories, L'Arbresle, France) and from C57BL/6 GCLM knock out mice (provided by Timothy P. Murphy, Department of Environmental Health, Center for Environmental Genetics, Cincinnati, OH and bred in our animal facility) in accordance with the authorization issued by the "Office Vétérinaire du Canton de Vaud". The pregnant female mouse was decapitated, brains of embryos were removed and cortices were isolated in Hanks' balanced salt solution (137mM NaCl, 5.3mM KCl, 0.45mM KH_2PO_4, 0.34mM Na_2HPO_4, 4.16mM $NaHCO_3$, 5.5mM glucose, 1mM sodium pyruvate, pH = 7.4) containing penicillin (100 U/ml) and streptomycin (100µg/ml). Cortical cells were mechanically dissociated with a Pasteur pipette, centrifuged and

resuspended in Neurobasal medium (Invitrogen) supplemented with 2% B27 (Invitrogen), 500µM glutamine and 30µM glutamate. GCLM KO female mice being unfertile, KO males were mated with heterozygote females to obtain KO and heterozygote fetuses. In these cases cortices dissected from fetuses could not be pooled and every brain had to be put in culture separately. Genotyping was done afterwards on animal's skin. Neurons were plated on poly-L-ornithine-coated culture dishes at a density of 800 cells/mm^2 or on poly-L-ornithine-coated glass coverslips at a density of 600 cells/mm^2. Cells were maintained at 37°C in a humidified 5% CO_2 atmosphere.

6.2.2 Primary cultures of cortical astrocytes

Cortices from P1-3 OF1 mice (S/IOPS OF1 from Charles River Laboratories, L'Arbresle, France) and C57BL/6 GCLM KO, heterozygotes (HZ) and wild-type (WT) mice were dissected in DMEM medium (Invitrogen, Carlsbad, CA, USA) supplement with 10% fetal bovine serum (Invitrogen) and containing penicillin (100 u/ml) and streptomycin (100µg/ml). Cortical cells were mechanically dissociated through needles with decreasing diameters, centrifuged at 500 rpm for 5 min 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.

6.2.3 Treatments

Unless otherwise cited, four to six days-old neurons and 14-15 days-old astrocytes, either from OF1 or from GCLM KO, HZ and WT mice, were exposed for 24h to various concentrations of curcumin, quercetin, tBHQ or FK506 (figure 20). All substances were diluted in DMSO with a final percentage of DMSO lower than 0.09%. Cells were put in the presence of the substances following a medium renewal. New medium was made of Neurobasal supplemented with 500 µM glutamine and 2% B27 without antioxidant and contained 5µM, 10µM, 20µM, 50µM or 100µM of substance or only DMSO as a control. These concentrations were chosen based on other *in vitro* studies using these compounds. For a given substance, treatments with the various concentrations and control were done on separate sister dishes.

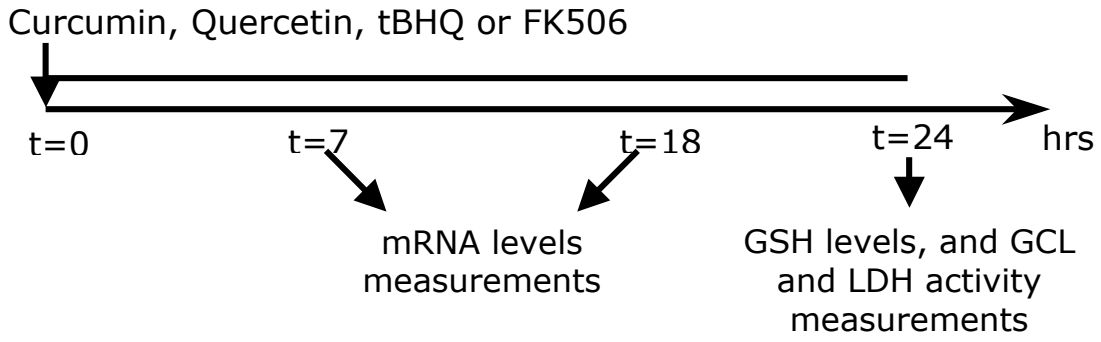


Figure 20. Experimental design

6.2.4 Oxidized and reduced GSH measurement

In order to assess the redox status in the cells, quantification of total GSH and oxidized GSH (GSSG) content in cells was performed with an assay based on the Tietze method (Tietze, 1969). Cells were washed twice with ice-cold PBS (150mM NaCl, 3mM KCl, 1.5mM KH_2PO_4 and 7.9mM Na_2HPO_4), and scrapped out of the dish with 100 µl ice-cold PBS containing 1-2.5mM EDTA. After sonication, an aliquot was reserved for subsequent analysis of protein content using the Advanced Protein Assay (Cytoskeleton, Denver, CO, USA). Proteins in the remaining cell solution were precipitated with 5-Sulfosalicylic acid (SSA) and precipitate was removed from solution by centrifugation. Triethanolamine (TEA) was added to the supernatant to bring pH >7. For GSSG measurement, a part of the supernatant was incubated with 2-vinylpyridine monomer (2VP) for 45 to 60 minutes at room temperature. 2VP forms a stable complex with reduced glutathione. In a well of a 96-well plate, 100µl of supernatant diluted in PBS as much as needed, was mixed with 100 µl of freshly-made reaction solution [PBS with 2.5mM EDTA, 0.3mM DTNB, 0.3mM NADPH and 1 U/ml GSH reductase]. The absorbance at 405 nm was measured with a plate reader 20 sec after mixing and then at 15 sec intervals for 1 min. The rate of increase in absorbance, which measures the cyclic reduction of DTNB by GSH, was proportional to the total GSH or to the amount of GSSG when 2VP was added. Measurements were done in duplicates. Concentration of GSH and GSSG in sample solution was determined by comparison with a series of freshly-made GSH and GSSG standard solutions respectively. Total GSH and GSSG content in cells are expressed in nmole GSH/mg proteins and nmole GSSG/mg proteins respectively. In order to better see the changes in GSSG relative to GSH, the percentage

of GSSG relative to GSH was calculated. In this case, GSH levels = total GSH levels – 2 x GSSG levels.

6.2.5 GCL activity measurements

GCL activity was measured with an assay based on (White et al., 2003). One 10-cm dish was used per treatment for astrocytes, while 3 to 6 60-mm dishes were necessary for neurons. Cells were washed twice with ice-cold PBS, and scrapped out of the dish with ice-cold PBS containing 1mM EDTA. Cells were freezed at – 80°C until assay. After sonication, the cell solution was centrifuged at 14000 RPM for 15 min. An aliquot from the supernatant was reserved for subsequent analysis of protein content using the Advanced Protein Assay. On a 96-well round bottom reaction plate, 50 µl of cell solution was put into eight wells, four of which already contained 5 µl of 40mM BSO. Plate was preincubated at 37°C for 15 min. 50 µl of reaction cocktail [stock solution made of : 400mM Tris, 20mM L-glutamic acid, 2mM EDTA, 20mM sodium borate, 2mM serine, plus 40mM ATP added freshly] was then added to each well. Five min later, reaction was initiated by adding 2mM cysteine [diluted in a stock solution made of: 20mM Tris, 1mM EDTA, 250mM D(+)-sucrose, 20mM sodium borate and 2mM L-serine]. The plate was then covered and incubated at 37°C for 45 min, time during which cysteine is linked to glutamine via GCL to form γ-glucys. The reaction was stopped when 200mM SSA was added to all wells and the plate was centrifuged for 5 min at 1200 g. 20 µl of supernatant from each well was transferred to a 96-well plate designed for fluorescence detection. A series of wells also contained various concentrations of freshly made GSH solution for standard curve. 200 µl of 10mM NDA solution was added to all wells [NDA was diluted in DMSO and 1 vol was added to 8 vol of a stock solution made of: 50mM Tris and 500mM NaOH, pH 12.5]. NDA forms a conjugate with the cysteine sulfhydryl group and the glutamyl amino group of γ-glucys and of GSH with the same affinity. After 30-min incubation at room temperature, fluorescence was measured with an excitatory wavelength of 485nm and an absorbance filter set to 530nm. No glycine was added to the reaction mix, so measured fluorescence is related to the levels of γ-glucys. Since equimolar of GSH and γ-glucys give similar NDA fluorescence, we used GSH for standard curves. Basal GSH levels measured in the well where GCL was blocked with BSO were subtracted to the values obtained where GCL was active. GCL activity is expressed in nmol GSH/mg proteins/min.

6.2.6 GST activity measurements

Total GST activity was assessed by measuring the conjugation of CDNB with reduced GSH (Habig et al., 1974), such a conjugation depending on GST. Cells were washed twice with ice-cold PBS, and scrapped out of the dish with ice-cold potassium phosphate buffer (PPB; pH 6.5) containing 2mM EDTA. After sonication, the cell solution was centrifuged at 14000 RPM for 15 min. Supernatant was freezed at – 80°C until assay. Once unfrozen, an aliquot from the supernatant was reserved for subsequent analysis of protein content using the Advanced Protein Assay. On a 96-well plate, 20 µl of cell solution was diluted into PPB containing 0.1% Triton. Freshly-made GSH was also added for a final concentration of 1mM in well. The reaction was initiated by adding CDNB and the absorbance was red at 340nm every 15 sec for 5 min. Measurements were done in triplicates. Activity of GST in sample solution was determined by comparison with a series of freshly-made GST standard solutions. The possible presence in samples of the substance used in the treatment did not interfere with the measurements of GST as verified using standard solutions of the various substances. GST activity in cells is expressed in U/mg proteins.

6.2.7 Gene expression

After 8-h or 18-h treatment, mRNA levels were measured in cultured neurons and astrocytes, with the use of TaqMan chemistry. mRNA was first extracted from samples with an RNA isolation kit (Promega, Madisson, WI, USA). Total mRNA was quantified using Ribogreen Assay kit (Invitrogen, Carlsbad, CA, USA) and 300 ng of total RNA was reverse-transcribed to cDNA. This cDNA was then amplified using TaqMan gene expression assays (Applied Biosystem, Foster City, CA, USA) at the following amplification condition: 1 cycle for 2 min at 50°C, 1 cycle for 10 min at 95°C, and 50 cycles for 15 s at 95°C, followed by 1 min at 60°C. The following genes were amplified: *Gclm* (Applied Biosystem, Mm00514996_m1), *Gclc* (mM00802655_m1), *Gpx1* (Mm00656767_m1), *Gss* (Mm00515065_m1), *Nrf2* (Mm00477784_m1), *Nrf3* (Mm00477788_m1), *xCT* (Mm00442530_m1) and *4F2* (Mm00500521_m1). Mouse 18S ribosomal protein (4319413E) was used as endogenous control. cDNA of treated sample was amplified again from original mRNA when the number of cycle to reach treshold (Ct) for 18S was different between treated and control samples on a same plate. Measurements were done in duplicates. Gene expression was calculated using the ΔΔCt

method (proposed by Applied Biosystem). ΔCt, that is the difference between Ct of 18S subtracted from Ct of the gene of interest, is calculated from both the treated and the control samples. ΔCt obtained with control is then subtracted from ΔCt obtained from treatment sample and linearity is achieved with the expression $2^{-\Delta\Delta Ct}$. The result obtained represents the amount of target gene in treated sample, normalized to an endogenous reference (18S) and relative to the control.

6.2.8 Assessment of cell viability

Cell viability was assessed by measuring the activity of lactate dehydrogenase (LDH) in the culture medium (adapted from Dringen et al., 1998). When cells are dying, cell membranes become permeable and cells release their content, including LDH, in the medium. An aliquot of culture medium was collected and kept at – 80°C. For measurement of LDH activity, 50 µl of the collected medium was mixed with 100 µl of 0.1M potassium phosphate buffer (pH 7.4) and 150 µl of a freshly-made reaction solution [3.2mM sodium pyruvate and 0.4mM NADH in 0.1 M potassium phosphate buffer]. The decrease in absorbance at 340 nm, proportional to LDH activity, was recorded every minute for 10 min using a plate reader. Measurements were done in duplicates. LDH activity in the medium was scaled with the activity of a series of LDH standard solutions. The amount of LDH in the medium was then reported to the amount of proteins measured in the cells of the corresponding dish.

6.2.9 Statistical analyses

The potential effect of DMSO, in which all substances are dissolved, was assessed by comparing cells put in the presence of medium containing or not DMSO. For all the measurements, t-tests showed no differences between DMSO and no DMSO situations. Therefore, DMSO situation is considered as the control. The effect of treatments versus control on GSH and GSSG content, on GCL and GST activity, on gene expression and on cell viability was assessed with the t-test for dependant samples. Statistical significance was corrected for multiple comparisons using the sequential Bonferroni test with the Dunn-Šidák method (Sokal and Rohlf, 1997), when 4 or 5 different concentrations were used. One-way multivariate analysis of variance (MANOVA) with Concentration as dependent and Substance as independent variable, was used to assess the difference between the effects induced by the various substances. The concentration

5µM was not included in the analyses, since this concentration was not used during tBHQ treatments. The same analysis, with Concentration as dependent and Cell type as independent variable, was used to look at the effect of cell types. When significance was achieved, post-hoc comparisons were performed. To assess the differential effects of a treatment on the three different genotypes, two-way ANOVA was used, with Genotype and Concentration as between factor, followed by a paired comparison with LS-means correction. For the effect of treatments on cell death, LDH activity in medium from dishes containing the different genotypes was compared between genotypes with a t-test for independent samples. LDH activity in medium from treated sample was compared with a t-test for dependant samples with LDH activity in medium from untreated cells of the same genotype. Significant probability level was set to $p \leq 0.05$.

6.3 Results

6.3.1 Curcumin

In neurons (figure 21), treatment with 5µM ($p < 0.05$), 10µM ($p < 0.001$), 20µM ($p < 0.05$), 50µM ($p < 0.01$) or 100µM ($p < 0.05$) curcumin significantly increased GSH levels of about 50%. No significant change was observed in the percentage of GSSG relative to total GSH, although a trend in an increase could be observed with 100µM. Because of material limitation, GCL activity was measured only after treatment with 10µM curcumin, concentration that was statistically the most efficient in increasing GSH levels. GCL activity was significantly increased of about 60% after this treatment ($p < 0.05$). No change in GST activity was observed, although a trend in an increase could be observed with 50µM ($p < 0.05$ before bonferonni correction). No increase in cell death was observed. Treatment with 10µM curcumin led to an increase in gene expression of xCT already after 8 hours and of the transcription factor Nrf2, the two subunits of the cystine-glutamate exchanger, xCT and 4F2, and the modulatory subunit of GCL, after 18h (table 2). The two genes that showed the greatest increase in their expression were GCLM and xCT.

In astrocytes (figure22), treatment with 20µM ($p < 0.001$), 50µM ($p < 0.001$) or 100µM ($p < 0.01$) curcumin significantly increased GSH levels to as much as 125%. A trend

was already observed with 10µM ($p < 0.05$ before bonferonni correction). There was no change in the content of GSSG relative to total GSH. The increase in GSH levels was accompanied by an increase in GCL activity with 10µM ($p < 0.05$), 20µM ($p < 0.05$), 50µM ($p < 0.01$) or 100µM ($p < 0.05$) curcumin. A significant decrease in GST activity was measured after treatment with 20µM ($p < 0.05$), 50µM ($p < 0.05$) or 100µM ($p < 0.05$ before bonferonni correction). No increase in cell death was observed. 18-h treatment with 10µM curcumin slightly increased gene expression of GCLM and GSS, while a treatment with the same duration but with 50µM led to an increase in mRNA levels of the transcription factor Nrf2, the two subunits of the cystine-glutamate exchanger, xCT and 4F2, the two subunits of GCL, GCLC and GCLM, and the second synthesizing enzyme of GSH, GSS (table 2). Like in neurons, the two genes that showed the greatest increase in their expression were GCLM and xCT, but increase in GSS mRNA was also important in astrocytes.

MANOVA on GSH levels data with Concentration as dependent variable showed a significant effect of cell type with 50µM curcumin ($p = 0.01$), meaning that the increase in GSH levels observed in astrocytes is significantly more important than in neurons.

See figure 23 for a summary of the results obtained following 24-h treatment with curcumin in cultured astrocytes and neurons.

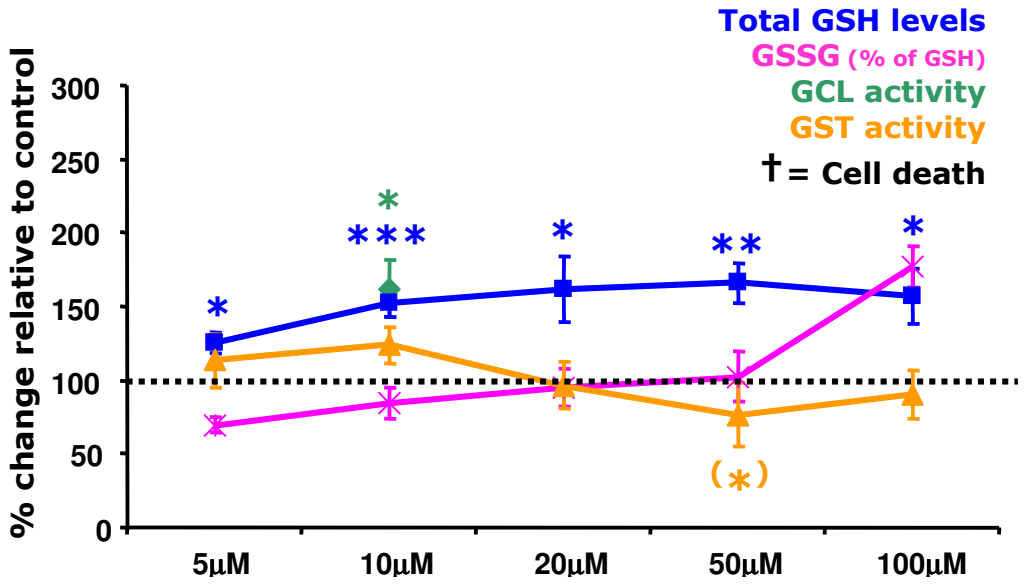


Figure 21. **Curcumin in neurons**. Effect of curcumin on GSH (n = 4 to 12) and GSSG (n = 3 or 4) levels, GCL (n = 7) and GST (n = 4 or 5) activity, and cell death (n= 12 to 14) in neurons. Data are expressed as percentage of change relative to control ± SEM. *, p<0.05; **, p<0.01; ***, p<0.001.

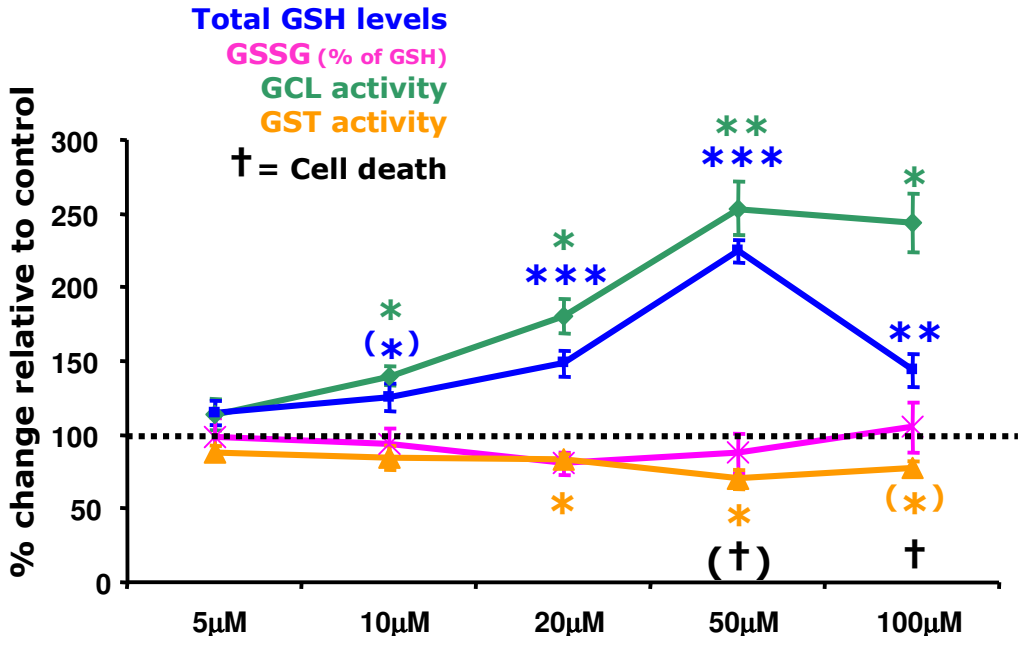


Figure 22. **Curcumin in astrocytes.** Effect of curcumin on GSH (n = 10 or 11) and GSSG (n = 5) levels, GCL (n = 4 to 7) and GST (n = 4) activity, and cell death (n= 12 to 13) in astrocytes. Data are expressed as percentage of change relative to control ± SEM. (*), p<0.05 before correction; *, p<0.05; **, p<0.01; ***, p<0.001.

	Neurones		Astrocytes	
Concentration of curcumin	10µM	10µM	10µM	50µM
Duration of treatment	8h	18h	18h	18h
Number of dishes	n=3	n=6	n=4	n=5
nrf2	1.39	**1.97**	0.97	**2.40**
xCT	**5.58**	**8.62**	0.87	**5.46**
4F2	1.33	**1.61**	0.87	**2.66**
gclc	1.05	1.79	1.04	**3.29**
gclm	1.68	**6.10**	**1.24**	**15.84**
gss	0.87	1.81	**1.29**	**5.62**
gpx1	0.64	1.24	1.01	1.41

Table 2. Effect of curcumin on gene expression in neurons and astrocytes. Results represent the amount of target gene in treated sample, normalized to an endogenous reference (18S) and relative to the control. **$p<0.05$**, **$0.05 < p < 0.01$**.

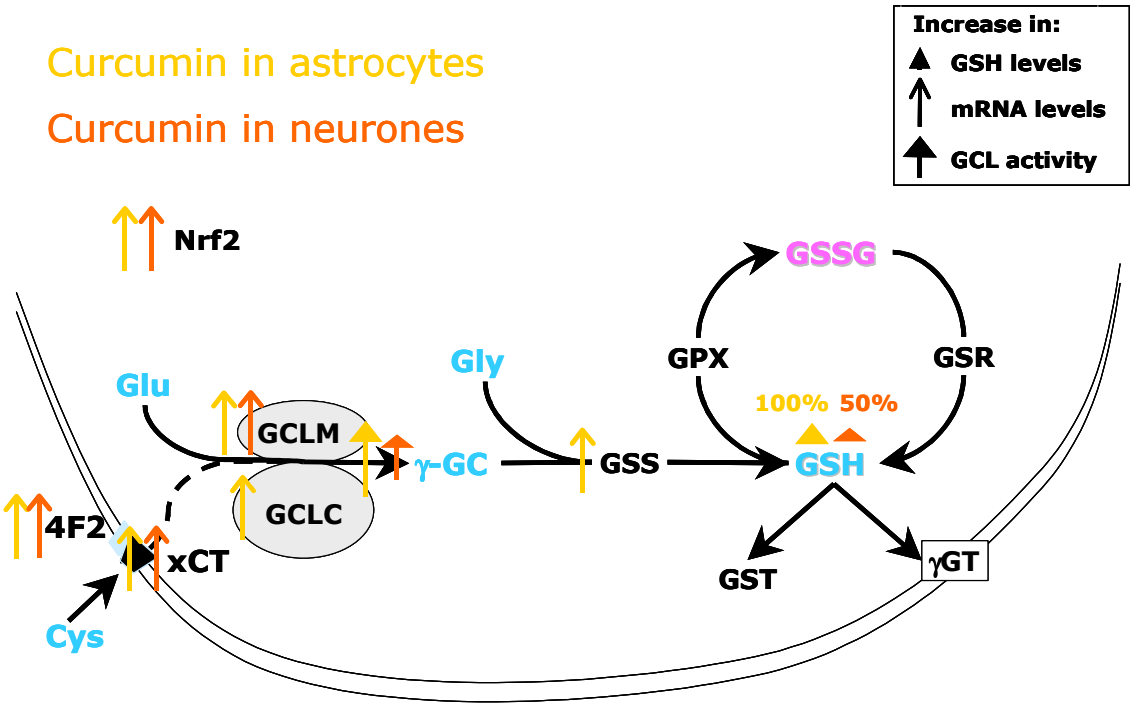


Figure 23. Summary of the results obtained following 24-h treatment with curcumin in cultured astrocytes and neurons. Only significant changes are shown.

6.3.2 Quercetin

In neurons (figure 24), treatment with low concentration of quercetin had no effect on GSH levels. Higher concentrations (50µM and 100µM) significantly decreased GSH levels ($p < 0.01$). Furthermore, cell death was significant with 50µM ($p < 0.05$) and nearly significant with 100µM ($p < 0.05$ before bonferonni correction), indicating a potential toxic effect of quercetin at high concentration in neurons. No significant change was observed in the percentage of GSSG relative to total GSH. Since quercetin did not succeed in increasing GSH levels in neurons, the effects of this substance on GCL and GST activity, and on mRNA levels were not evaluated.

In astrocytes (figure 25), treatment with 10µM ($p < 0.05$), 20µM ($p < 0.001$), 50µM ($p < 0.001$) or 100µM ($p < 0.01$) quercetin significantly increased GSH levels of about 50%. There was no change in the content of GSSG relative to total GSH. The increase in GSH levels was accompanied by an increase in GCL activity that was nearly significant with 5µM ($p < 0.05$ before bonferonni correction), and fully significant with 10µM ($p < 0.01$), 20µM ($p < 0.05$) or 100µM ($p < 0.01$) quercetin. No change in GST activity and no increase in cell death were observed. 8-h treatment with 20µM quercetin increased gene expression of xCT and GCLM, and to a lesser extend GSS. 18-h treatment with the same concentration also led to an increase in mRNA levels of xCT, GCLM and GSS, but also those of GCLC (table 3). Like with curcumin treatment, mRNA levels that were increased the most with quercetin were those of GCLM and xCT.

MANOVA on GSH levels data with Concentration as dependent variable showed a significant effect of cell type with 20µM ($p < 0.001$), 50µM ($p < 0.001$) and 100µM ($p < 0.001$) quercetin. Indeed these concentrations increased GSH levels in astrocytes, but not in neurons, where even a decrease was observed with the two higher concentrations.

See figure 26 for a summary of the results obtained following 24-h treatment with quercetin in cultured astrocytes and neurons.

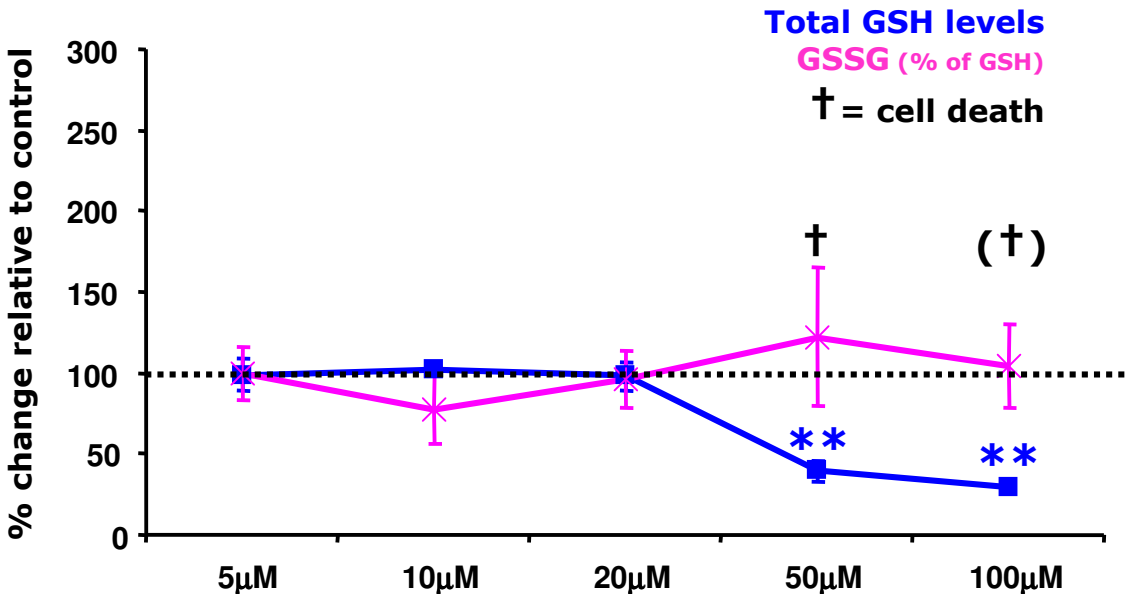


Figure 24. **Quercetin in neurons.** Effect of quercetin on GSH (n = 8) and GSSG (n = 4) levels, and cell death (n= 10 or 11) in neurons. Data are expressed as percentage of change relative to control ± SEM. (*), p<0.05 before correction; *, p<0.05; **, p<0.01; ***, p<0.001.

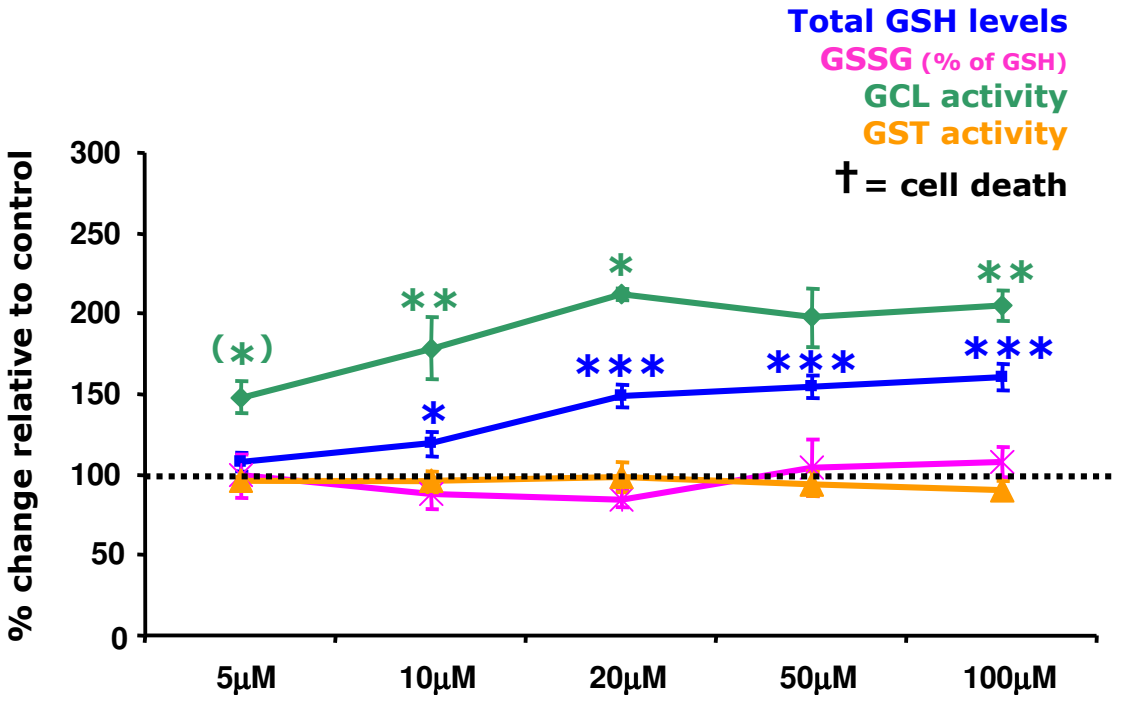


Figure 25. **Quercetin in astrocytes.** Effect of quercetin on GSH (n = 10 to 12) and GSSG (n = 5) levels, GCL (n = 5 to 7) and GST (n = 4) activity, and cell death (n= 11 to 13) in astrocytes. Data are expressed as percentage of change relative to control ± SEM. (*), p<0.05 before correction; *, p<0.05; **, p<0.01; ***, p<0.001.

	Astrocytes	
Concentration of quercetin	20µM	20µM
Duration of treatment	8h	18h
Number of dishes	n=4	n=4
nrf2	0,83	1,37
xCT	8,51	5,47
4F2	1,30	1,41
gclc	1,68	3,27
gclm	10,92	14,34
gss	2,05	3,70
gpx1	1,01	1,69

Table 3. Effect of quercetin on gene expression in astrocytes. Results represent the amount of target gene in treated sample, normalized to an endogenous reference (18S) and relative to the control. **p<0.05**, **0.05 < p < 0.01.**

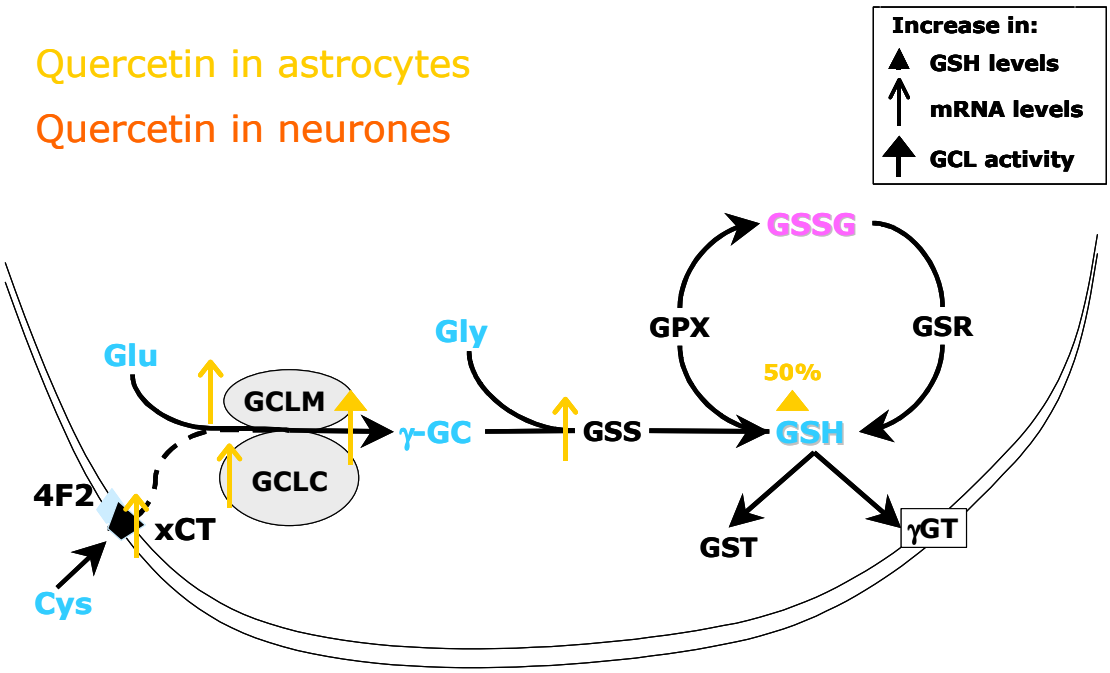


Figure 26. Summary of the results obtained following 24-h treatment with quercetin in cultured astrocytes and neurons. Only significant changes are shown.

6.3.3 *Tert*-Butylhydroquinone

In neurons (figure 27), treatment with 10µM ($p < 0.05$) and 20µM ($p < 0.01$) tBHQ significantly increased GSH levels of about 20%. tBHQ had no effect, while 100µM significantly decreased GSH levels ($p < 0.001$). This decrease was accompanied by an

increase in cell death that was already significant with 50µM ($p < 0.05$) and still observable with 100µM ($p < 0.05$). No significant change was observed in the percentage of GSSG relative to total GSH. Because of material limitation, GCL activity was measured only after treatment with 20µM tBHQ, concentration that was statistically the most efficient in increasing GSH levels. A trend in an increase of about 40% in GCL activity was observed, but it was not significant probably due to the small sample. No change in GST activity and no increase in cell death were observed. Treatment with 20µM tBHQ led to an increase in gene expression of xCT with a trend for GCLM already after only 8 hours, and of Nrf2, xCT, 4F2, GCLC, GCLM and GSS after 18h (table 4). The genes that showed the highest increase in their expression were GCLM, xCT, and GSS.

In astrocytes (figure 28), treatment with 20µM ($p < 0.001$), 50µM ($p < 0.001$) tBHQ significantly increased GSH levels of about 50%. This increase was nearly significant with 10µM ($p < 0.05$ before bonferonni correction), while no change was observed with 100µM. At this concentration a trend in cell death was present ($p < 0.05$ before bonferonni correction). There was no change in the content of GSSG relative to total GSH, although a trend in an increase could be observed with 100µM. The increase in GCL activity was greater than that in GSH levels, with values as much as 150% higher than in controls. This increase was significant with 10µM ($p < 0.01$), 20µM ($p < 0.01$), 50µM ($p < 0.001$) or 100µM ($p < 0.01$) tBHQ. No change in GST activity was observed. 8-h treatment with 100µM tBHQ slightly increased gene expression of GSS, but already had a huge effect on mRNA levels of xCT and GCLM. 18-h treatment with the same concentration led to an increase in mRNA levels of xCT, 4F2, GCLC, GCLM, GSS and GPX (table 3). Like in neurons, the genes that showed the highest increase in their expression were GCLM, xCT, and GSS, with values as high as 20x to 40x higher relative to controls.

MANOVA on GSH levels data with Concentration as dependent variable showed a significant effect of cell type with 100µM tBHQ ($p = 0.006$). Indeed, while GSH levels were increased in astrocytes, they were diminished in neurons.

See figure 29 for a summary of the results obtained following 24-h treatment with tBHQ in cultured astrocytes and neurons.

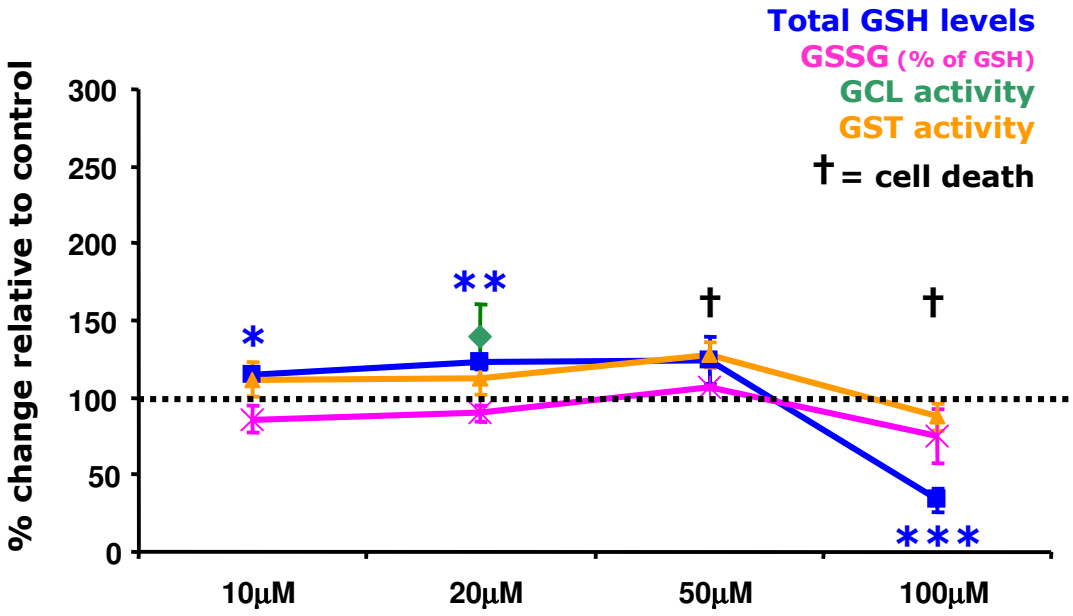


Figure 27. **tBHQ in neurons**. Effect of tBHQ on GSH (n = 7 to 9) and GSSG (n = 3) levels, GCL (n = 4) and GST (n = 4) activity, and cell death (n= 10 to 13) in neurons. Data are expressed as percentage of change relative to control ± SEM. (*), p<0.05 before correction; *, p<0.05; **, p<0.01; ***, p<0.001.

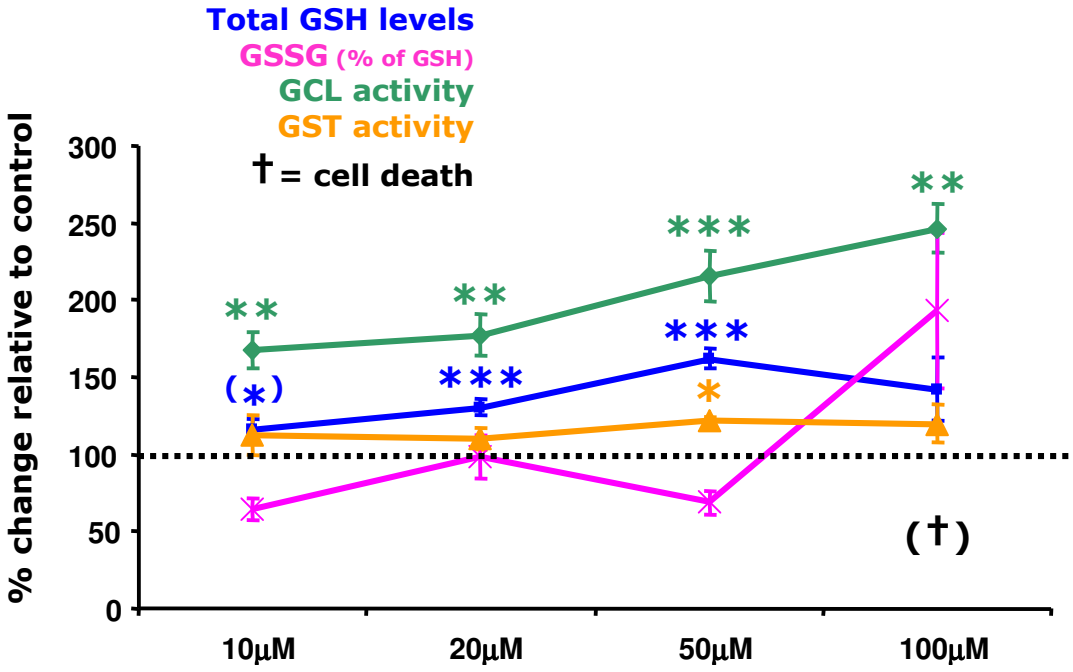


Figure 28. **tBHQ in astrocytes.** Effect of tBHQ on GSH (n = 9 to 12) and GSSG (n = 5) levels, GCL (n = 6 or 7) and GST (n = 5) activity, and cell death (n= 14 to 17) in astrocytes. Data are expressed as percentage of change relative to control ± SEM. (*), p<0.05 before correction; *, p<0.05; **, p<0.01; ***, p<0.001.

	Neurones		Astrocytes	
Concentration of tBHQ	20µM	20µM	100µM	100µM
Duration of treatment	8h	18h	8h	18h
Number of dishes	n=3	n=5	n=5	n=4
nrf2	1,27	3,49	0,91	1,86
xCT	6,57	15,03	71,62	38,92
4F2	0,99	2,60	2,73	4,65
gclc	1,42	3,15	2,58	6,51
gclm	1,66	9,47	29,75	42,82
gss	1,14	5,10	4,62	20,46
gpx1	0,86	2,71	1,05	4,81

Table 4. Effect of tBHQ on gene expression in neurons and astrocytes. Results represent the amount of target gene in treated sample, normalized to an endogenous reference (18S) and relative to the control. **p<0.05**, **0.05 < p < 0.01.**

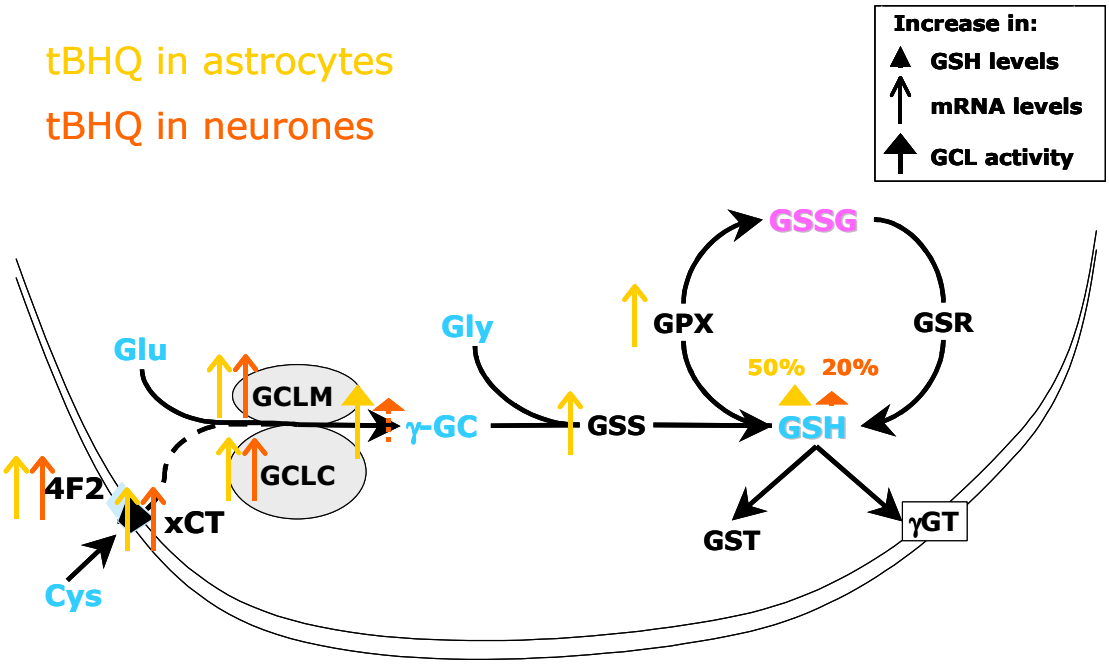


Figure 29. Summary of the results obtained following 24-h treatment with tBHQ in cultured astrocytes and neurons. Only significant changes are shown.

Statistical analyses allowing the comparison between substances were performed. In neurons, MANOVA on GSH levels data with Concentration as dependent variable showed a significant effect of substance. Treatment with curcumin was more efficient than tBHQ and quercetin in increasing GSH levels at a concentration of 10µM. At 20µM and 50µM tBHQ was more efficient than quercetin, but curcumin was still the best treatment. Finally, with a concentration of 100µM, curcumin was still increasing GSH levels, while the two other substances led to a decrease in GSH levels.

In astrocytes, the same analysis also showed a significant effect of substance, but only at a concentration of 50µM. Indeed, at this concentration, treatment with curcumin was more efficient than tBHQ and quercetin in increasing GSH levels. All three substances were equally efficient at other concentrations.

6.3.4 FK506

In both neurons (figure 30) and astrocytes (figure 31), FK506 showed no capacity in increasing GSH levels, nor did it lead to cell death. No change in GSH levels being observed, this substances was not further investigated.

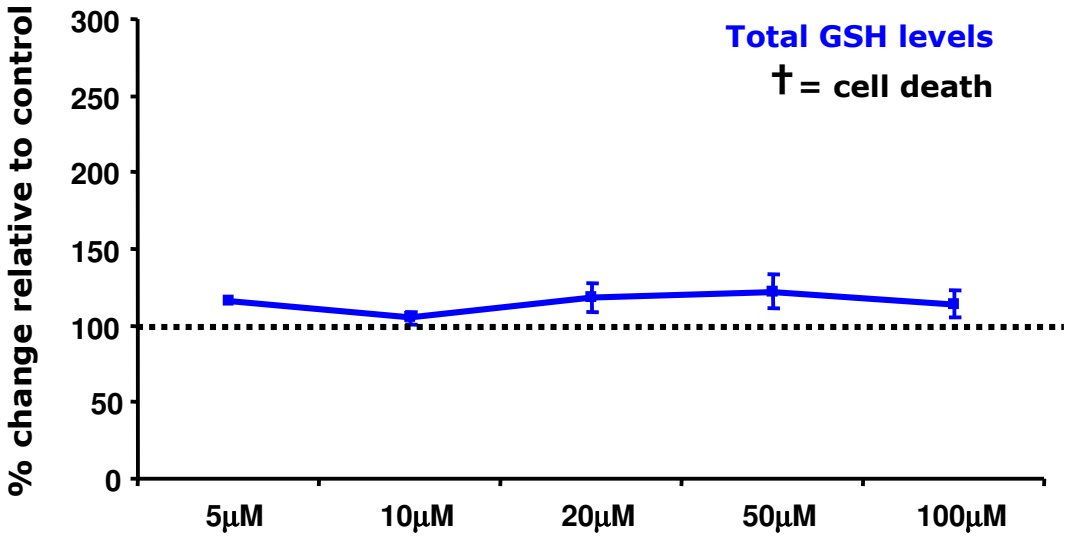


Figure 30. **FK506 in neurons.** Effect of FK506 on GSH levels (n = 2) in neurons. Data are expressed as percentage of change relative to control ± SEM.

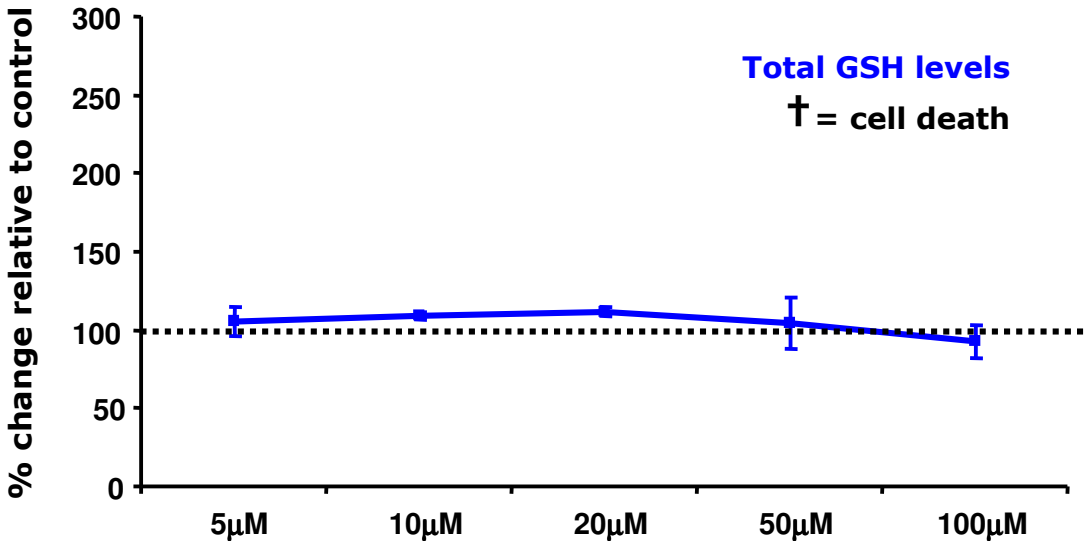


Figure 31. **FK506 in astrocytes.** Effect of FK506 on GSH levels (n = 2 or 3) in astrocytes. Data are expressed as percentage of change relative to control ± SEM.

6.3.5 Effects of treatments in astrocytes from GCLM KO mouse

The enzyme GCL is implicated in the pathophysiology of schizophrenia. The catalytic activity of the subunit GCLC is modulated by the subunit GCLM. Polymorphisms in the gene of the modulatory subunit of GCL (GCLM) was shown to be associated with schizophrenia in three different populations and GCLM gene expression is decreased in fibroblasts of patients (Tosic et al., 2006). After treatment with curcumin, quercetin and tBHQ, GCLM gene was always the most upregulated, stressing the importance of this subunit in the induction of GCL. The availability of the GCLM knockout mouse allowed us to investigate the necessity of this subunit in the action of the substances used.

Figure 32 shows basal GSH levels in cultured neurons from wildtype (WT), heterozygous (HZ) and GCLM knockout (KO) mice. No significant difference in GSH levels could be measured between WT and HZ, while, in KO, these levels were decreased by 74% compared to WT ($p < 0.001$). However, no increase in cell death was measured in neurons from KO. Due to material limitation, basal GCL activity was not measured in these neurons and treatment with substances were done only in astrocytes.

In astrocytes (figure 33 and 34), no significant difference in both GSH levels and GCL activity was observed between WT and HZ. Surprisingly, while GSH levels in KO were decreased by 80% compared to WT ($p < 0.001$), the 31% decrease in GCL activity was not significant. No increase in cell death was observed in astrocytes from KO.

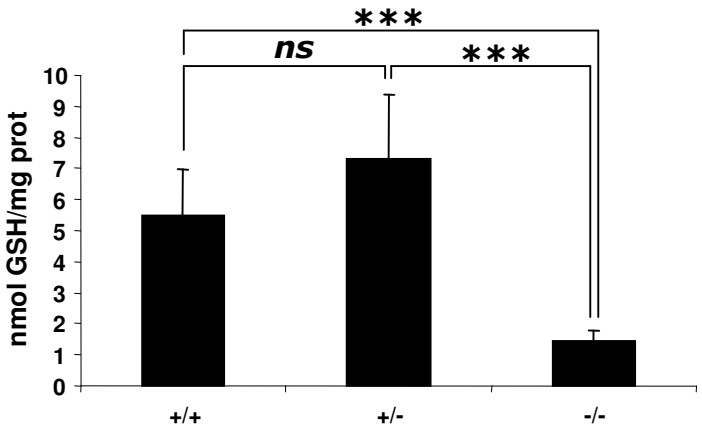


Figure 32. Basal GSH levels in neurons from GCLM WT (n=4), HZ (n=3) and KO mice (n=6). ***, $p<0.001$.

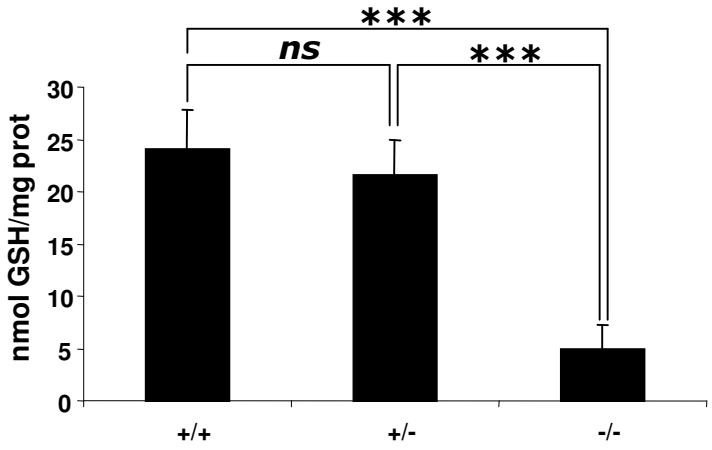


Figure 33. Basal GSH levels in astrocytes from GCLM WT (n=11), HZ (n=17) and KO (n=11) mice. ***, p<0.001.

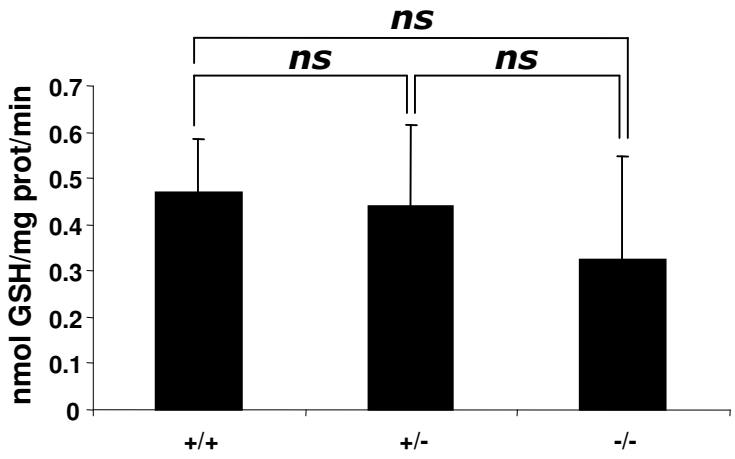


Figure 34. Basal GCL levels in astrocytes from GCLM WT (n=5), HZ (n=23) and KO (n=11) mice.

6.3.5.1 Curcumin

In wild-type (WT), like in OF1 (figure 21), a trend in an increase in GSH levels was observed with 10µM curcumin and 50µM increased GSH levels by more than 125% ($p < 0.001$; figure 35A). This was accompanied by an increase in GCL activity that was significant only with a concentration of 50µM (figure 35B). Treatment with 50µM curcumin also increased GSH levels in HZ ($p < 0.001$; figure 35A), but significantly less than in WT ($p < 0.01$). Surprisingly, the increase in GCL activity induced by 50µM curcumin was significantly higher in HZ (200%; $p < 0.001$) compared to WT (120%; $p < 0.05$; figure 35B). This could just be due to the fact that the number of sample was higher in HZ (n=9) than in WT (n=5), leading to a higher variance in the last type of cells. In astrocytes from KO, neither 10µM nor 50µM could increase GSH levels and GCL activity (figure 35). Furthermore, treatment with 50µM led to significant cell death ($p < 0.05$).

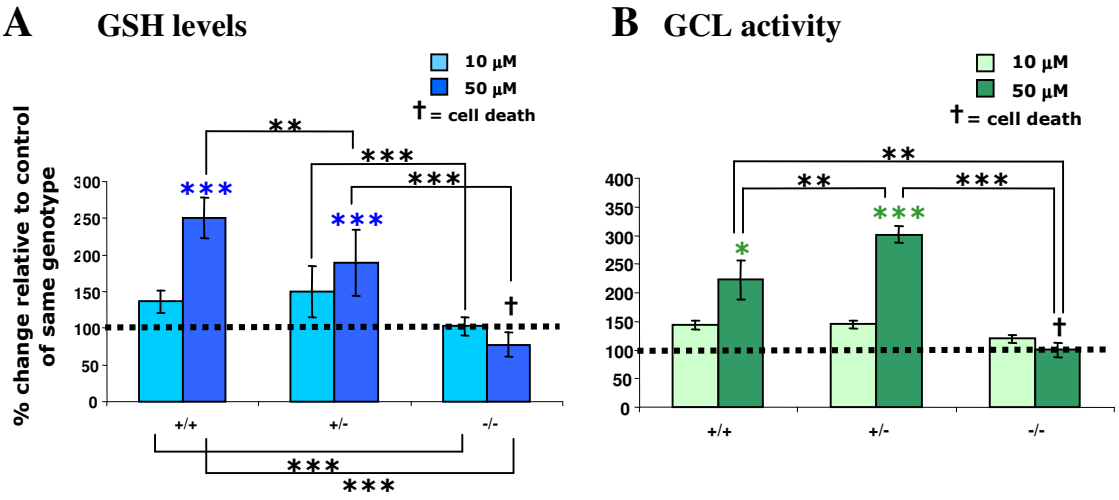


Figure 35. Effect of curcumin on GSH levels (A; n=4 to 13), GCL activity (B; n=2 to 17) and cell death (n=5 to 13) in astrocytes from GCLM WT, heterozygous and KO mice. *** or ***, p<0.001; compared to control of same genotype.
*, p<0.05; **, p<0.01; ***, p<0.001; compared to other genotype after treatment with the same concentration.

6.3.5.2 Quercetin

In WT, like in OF1 (figure 25), an increase in GSH levels of more than 50% was observed with 20µM quercetin ($p < 0.001$; figure 36A). This was accompanied by a 200% increase in GCL activity ($p < 0.001$; figure 36B). Treatment with 20µM quercetin also increased GSH levels in HZ ($p < 0.01$; figure 36A). The increase in GCL activity induced by 20µM quercetin was equivalent to that observed in WT ($p< 0.01$; figure 36B). In astrocytes from KO, 20µM quercetin treatment could not increase neither GSH levels, nor GCL activity (figure 36), but no cell death was observed at this concentration.

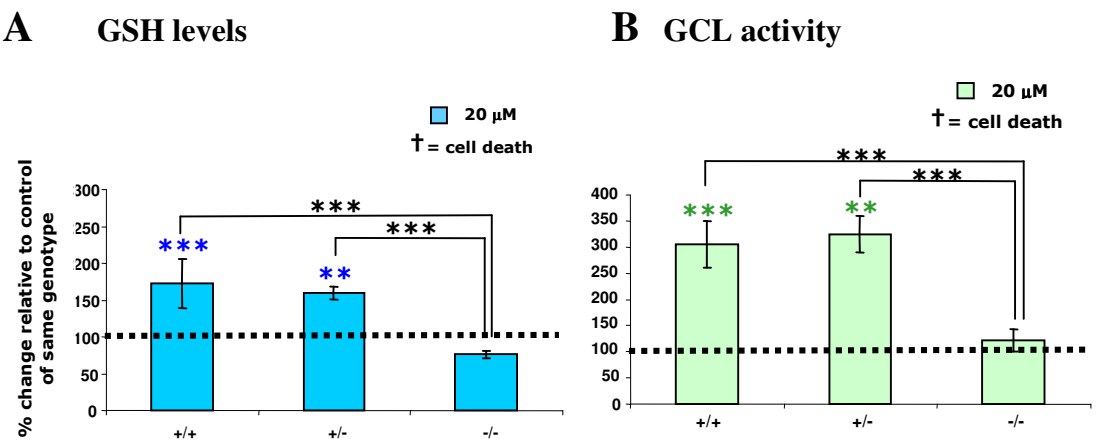


Figure 36. Effect of quercetin on GSH levels (A; n=3 or 4), GCL activity (B; n=3 or 4) and cell death (n= 3 or 4) in astrocytes from GCLM WT, heterozygous and KO mice. (*), $p<0.05$ before correction; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

6.3.5.3 tBHQ

In WT, like in OF1 (figure 21), a trend in an increase in GSH levels was observed with 20µM tBHQ. With 100µM, GSH levels were increased by about 100% ($p < 0.001$; figure 37A), while at this concentration GSH levels were not increased anymore in OF1. Increase in GCL activity was significant only with a concentration of 100µM ($p < 0.05$; figure 37B). Treatment with 100µM tBHQ increased GSH levels ($p < 0.001$; figure 37A) and GCL activity in HZ ($p < 0.05$). The trend in an increase of GCL activity with 20µM tBHQ in both WT and HZ was not significant. In astrocytes from KO, neither

20µM nor 100µM tBHQ could increase GSH levels and GCL activity (figure 34). Furthermore, treatment with 100µM led to significant cell death ($p < 0.05$).

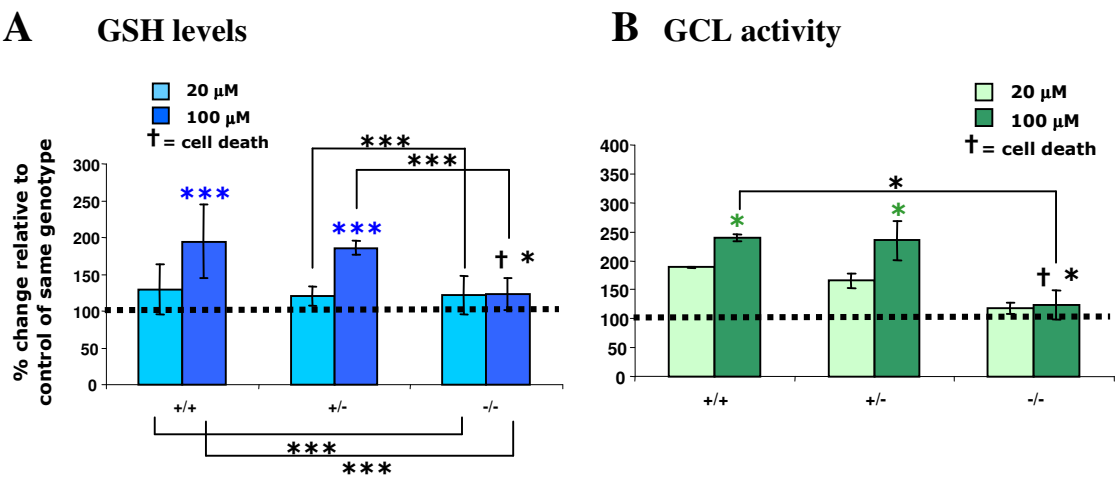


Figure 37. Effect of tBHQ on GSH levels (A; n=4 to 6), GCL activity (B; n=2 to 6) and cell death (n=4 to 6) in astrocytes from GCLM WT, heterozygous and KO mice. (*), $p<0.05$ before correction; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

7. Strategy 3 : Providing more of the limiting precursor cysteine

7.1 Introduction

Schizophrenia patients present deficits in their evoked-related potentials measured with the EEG technique during oddball paradigms. During these paradigms, participants are presented with two stimuli, usually auditive or visual, that are characterized by different physical properties (e.g. different frequency or duration). The participant is required to discriminate between the two stimuli, thus eliciting early waves (P50, N100) that are associated with pre-attentive processes and later components (P300), reflecting cognitive function. The amplitude of the P300 was shown to be decreased in schizophrenia already 30 years ago (Roth and Cannon, 1972) and these results were repeated several times (for a review see Hansenne, 2000). There is increasing evidence that the pathophysiology of schizophrenia entails both high-level cognitive impairments as well as (and perhaps as a consequence of) low-level sensory processing deficits across sensory modalities (Butler and Javitt, 2005;Foxe et al., 2005;Javitt et al., 1999). Indeed, There is a large body of evidence suggesting that a significant proportion of patients with schizophrenia have sensory gating impairments as reflected by a decrease

in P50 amplitude (for a review see Potter et al., 2006). Furthermore, previous research has consistently shown that the mismatch negativity (MMN) is impaired in schizophrenia patients (Catts et al., 1995;Javitt et al., 1993;Javitt et al., 1998;Shelley et al., 1991;Shutara et al., 1996). The auditory MMN is defined as the differential response to deviant stimuli compared to distractors. It is a wave that appears around 100ms after the presentation of a deviant, attended or unattended, stimulus. MMN generation is thought to rely on intact NMDA receptor (NMDA-R) function. Indeed, NMDA-R antagonists block the generation of this wave in both primates (Javitt et al., 1996) and humans (Umbricht et al., 2000), suggesting that MMN reflects selective current flowing through open, unblocked NMDA channels. The amplitude of MMN is known to be decreased in schizophrenia, indicating an impaired NMDA-R activity in these patients. Interestingly, the greater the MMN impairment, the lower are the Global Assessment of Functioning Scale ratings, suggesting that MMN is linked to global impairments in everyday functioning in schizophrenia patients (Light and Braff, 2005).

Several studies have demonstrated that the administration of NMDA-R antagonists, such as phencyclidine (PCP) or ketamine, to control subjects lead to the apparition of symptoms similar to those observed in schizophrenia and exacerbates these symptoms in patients (Krystal et al., 1994). It has been proposed that impairments in NMDA-R activity may contribute to the pathophysiology of schizophrenia (Coyle, 2006;Javitt and Zukin, 1991). The observed deficit in GSH in schizophrenia (Do et al., 2000) and the hypofunction of NMDA-R induced by low GSH (Steullet et al., 2006;Steullet et al., submitted), suggest that the dysregulation of the GSH system could be implicated in the reduced activity of NMDA-R in schizophrenia patients. Indeed, it was shown that GSH, as a reducing agent, can potentiate the activity of redox sensitive proteins, such as NMDA-R (Choi and Lipton, 2000;Kohr et al., 1994).

We propose that, by increasing GSH levels in the brain of schizophrenia patients, various aspects of the cerebral function, reflected by MMN (Light and Braff, 2005), could be improved. Therefore, as a last strategy to increase GSH levels, more cysteine, which is the rate-limiting precursor in the synthesis of GSH (Meister et al., 1986), will be provided to schizophrenia patients.

7.1.1 NAC

The free amino acid cysteine does not represent an ideal delivery system to the cell as it is potentially toxic and is spontaneously catabolized in the gastrointestinal tract and blood plasma. N-acetyl-cysteine (NAC) has therefore been used in various studies as a cysteine donor. Given orally, NAC is quickly absorbed and plasma peak concentration of cysteine are reached within 120 minutes (Borgstrom et al., 1986;Borgstrom and Kagedal, 1990;Olsson et al., 1988). NAC crosses the blood-brain barrier (Farr et al., 2003) and cysteine can be used in the brain as GSH precursor. Indeed, animal studies have shown that systemic administration of NAC protects brain against GSH depletion (Aydin et al., 2002;Ercal et al., 1996;Fu et al., 2006;Kamboj et al., 2006).

In the present study, NAC was used in an attempt to increase GSH levels in brain of schizophrenia patients. The effects of such GSH system modulation on the MMN as well as on the neuropsychological and biochemical status of schizophrenia patients were investigated.

7.2 Methods

7.2.1 Clinical trial

NAC (1g 2x/day) and placebo were administered to schizophrenia patients in a double-blind, cross-over design. One group received NAC for the first two months and then placebo for another period of two months, and the other group received placebo first and then NAC. NAC was purchased from Zambon (Italy). NAC and placebo capsules were manufactured by DFC Thompson (Sydney, Australia) and re-conditioned by a pharmacist of the Department of Psychiatry of the Centre Hospitalier Universitaire Vaudois and University of Lausanne. NAC being a precursor of GSH, we hypothesized that increasing brain levels of GSH in schizophrenia patients would improve cerebral functioning and in particular MMN generation. Electroencephalographic (EEG) recordings, and blood sampling were performed at the onset of the protocol (baseline measurements), at the point of cross-over, and at the end of the study. Psychopathological scales were evaluated every two weeks, according to the main trial protocol; the results of which are reported elsewhere (Berk et al., submitted).

7.2.2 Participants

Eleven patients (9 men; 2 women; aged 31.9±3.4yrs.; mean±SEM) meeting DSM-IV criteria for schizophrenia were recruited from the ambulatory Schizophrenia Service of the Department of Psychiatry of the Centre Hospitalier Universitaire Vaudois by an experienced psychiatrist and a psychologist well-trained in Diagnostic Interview for Genetic Studies. The mean duration of illness was 9.4±2.5yrs. Data from these patients at the onset of the protocol were compared with those from eleven sex-matched and age-matched healthy controls (9 men; 2 women; aged 34.4±2.9yrs.), who were selected on the basis that they had never suffered from any psychiatric disorder.

Patient	Age(y)/Sex/ Handedness	Length of Illness (y)	Past Medication	Current Medication	Lifetime diagnosis of drug abuse or dependence	Lifetime diagnosis of alcohol abuse or dependence
2	38/M/L	14	Haldol	Risperdal	No	Yes
3	46/M/R	3	Drug naive	Drug free	No	No
4	50/M/L	10	Truxal; Haldol	Risperdal; Fluctine; Temesta	No	No
5	37/M/R	10	Clopixol; Haldol	Risperdal; Selipram; Temesta ; Tranxilium	No	No
6	35/F/R	14	Fluanxol	Seruquel; Tranxilium; Imovane; Centrum;	No	No
9	51/M/R	29	Nozinan; Saroten; Dapotum; Melleril	Leponex; Zoloft	Yes	No
13	35/F/R	15	Fluctine	Zyprexa; Inderal	No	No

Table 5. Demographic and clinical characteristics of participants.

Among the eleven patients, nine participated in the clinical trial and seven completed the entire study, including EEG recordings at cross-over and at the end of the study (see Table 5). The two patients who withdrew from the study reported that it was too demanding for them. Of the seven patients who completed the entire study, five were among the group that first received NAC and then placebo; the remaining two received placebo first and then NAC. As such, any effects observed during NAC administration are unlikely to follow simply from repeated task performance. Following their recruitment, patients were given an ID number, and both patients and investigators were blinded until the time of analysis, when data pooling necessitated unblinding. Patients and controls were recruited with fully informed written consent, and all procedures were approved by the Ethics Committee of the Faculty of Biology and Medicine of the University of Lausanne. All eleven patients and eight of the healthy controls were

interviewed with the Diagnostic Interview for Genetic Studies, developed by the NIMH (Nurnberger et al., 1994;Preisig et al., 1999).

7.2.3 EEG Experimental paradigm

During the EEG recordings, participants were presented with auditory pure tone stimuli (50ms duration; 10ms rise/fall time; 44.1kHz sampling rate; 70dB SPL at the ear) and performed a go/no-go oddball discrimination task. On the majority of trials (80%), the pitch was 1kHz, and on the remaining 20% it was 2kHz. Stimuli were presented binaurally via insert earphones (model ER-4P; Etymotic Research, Inc., Elk Grove Villlage, USA), and the inter-stimulus interval was 1.5sec. Participants were instructed to perform a button press upon hearing the 2kHz tone (deviant) and to withhold responses to the 1kHz tone (standard). Stimulus presentation and response recording were controlled by E-prime (Psychology Software Tools, Inc., Pittsburgh, USA).

7.2.4 EEG recording and analyses

Continuous EEG was recorded at 500Hz (baseline measurements) or 1000Hz (cross-over and at the end of the protocol) through a 128-channel Geodesic Sensor Net system (Electrical Geodesics Inc., Eugene, OR) referenced to the vertex (Cz) and 100Hz low-pass filtered. Electrode impedance was maintained below 50kΩ. Peri-stimulus epochs of continuous EEG (100ms before to 600ms after stimulus onset) were averaged from each participant separately for the two different stimuli (i.e. standards and deviants) to compute AEPs. Epochs with muscle activity, eye movements, or other noise transients exceeding ±100μV were automatically rejected off-line. Data from artifact electrodes were interpolated (Perrin et al., 1987), and AEPs were down-sampled to a common 121-channel montage and baseline-corrected using the 100ms pre-stimulus period. AEPs were then recalculated to the common average reference and group-averaged.

For baseline recordings, AEPs from control subjects were based on 316±24 (mean±SEM) standard and 73±5 deviant stimuli, whereas those from patients were based on 590±57 standard and 148±14 deviant stimuli. Patients were presented with a larger number of stimuli to minimize the possibility that differences between populations followed from a lower signal-to-noise ratio in patients. The number of trials accepted in response to standard and deviant stimuli did not differ between recordings

during NAC and placebo treatments. Following NAC treatment, 726±39 standard and 179±9 deviant trials were included. Following placebo treatment, 626±95 standard and 164±18 deviant trials were included.

Two separate series of AEP analyses were conducted. The first followed a between-subjects design and was done in order to verify that our cohort of patients exhibited impaired MMN relative to healthy controls at the onset of the protocol, as has been repeatedly demonstrated in prior studies of schizophrenic patients (Catts et al., 1995;Javitt et al., 1993;Javitt et al., 1998;Shelley et al., 1991;Shutara et al., 1996). The second followed a within-subjects design and was done in order to assess whether two months of NAC administration restored MMN generation relative to the administration of placebo. AEPs were analyzed following methods described in detail elsewhere that permit the examination of both local and global features of the electric field at the scalp (Murray et al., 2004). We briefly summarize these methods here.

First, AEP components were identified using a topographic pattern analysis that is based on a modified atomize and agglomerate hierarchical clustering procedure as implemented in Cartool software (http://brainmapping.unige.ch/cartool.htm; see also Tibshirani et al., 2005). This clustering procedure identifies the topographies (i.e. maps) dominating the group-averaged AEPs across populations/conditions. The pattern observed at the group-average level was then statistically assessed at the individual participant level using a fitting procedure based on spatial correlation (Brandeis et al., 1995; see also Murray et al, 2006 for a recent publication of formulae), yielding a measure of map presence. These values are then submitted to ANOVA, revealing whether and when different maps explain responses from different populations/conditions and by extension whether and when different configurations of underlying sources are active, because topographic changes forcibly follow from different configurations of underlying brain networks (Lehmann, 1987). As this analysis identified the same set and sequence of topographies in AEPs from both patient and control groups as well as all experimental conditions, we do not discuss it in detail in the Results. The main utility of the topographic pattern analysis in the present study was in identifying the onset and offset of AEP components according to topographic stability in time (see Results for details). In particular, we focus on the N1 component, which in all cases was identified over the 70-155ms interval, given the abundant

evidence that the MMN is contemporaneous with the N1. In addition to AEP waveforms at specific electrode locations (which were selected based on the N1 topography), we also analyzed the strength of the electric field at the scalp using global field power (GFP) (Lehmann and Skrandies, 1980). GFP is equivalent to the root mean square across the electrode montage. AEP and GFP waveform area measures (vs. the 0µV baseline) from each stimulus condition over the 70-155ms interval were compared between controls and patients at protocol onset, and between NAC and placebo treatments.

7.2.5 Neuropsychological assessments and analyses

We used a computerized neuropsychological tests battery focused on the cognitive functions known to be impaired in schizophrenia; including attention, memory, executive functions and binding capacities. Sustained attention was assessed with the Degraded Stimulus Continuous Performance Task (DS-CPT; Nuechterlein, 1991). The Span of Apprehension Test (SOA; Asarnow and Granholm, 1991) during which the patient has to detect the letters F and T among twelve other letters, was administered as a selective attention task. Reaction time was measured both during the AOP and during a simple button-pressing task, where the patient had to press a button as soon as a black circle appeared in the center of the screen. A subscale of the Wechsler Adult Intelligence Scale (WAIS), that is the digit recall, was used to assess working memory, and visuo-spatial working memory was measured with the Plane-Flightball Task (Cocchi et al., 2007). As a planification task, we used three computerized items of the Hanoï Towers, the first trial being a practice, while the second and third ones, more difficult, were used for the analyses. Intra-modal binding capacity (Singer, 1995;Uhlhaas and Singer, 2006) was tested in a visual perception of shapes task (Parnas et al., 2001). These measures were assessed by a two-way, mixed effects ANOVA for crossover design with terms for Period (sequence), Patient and Treatment. The relevant effect of the treatment was adjusted for the random effect of the patient and for the study period (linked to the treatment sequence).

7.2.6 Biochemical measurements and analyses

GSH levels were measured in blood cells and GSH-related thiols, cysteine and cysteinyl-glycine, were measured in plasma. Blood was collected by venipuncture

between 7:00 and 8:30AM under restricted activity conditions and after fasting from the previous midnight. 18-20ml blood was collected in Vacutainer tubes coated with Ethylenediaminetetraacetic acid (Becton Dickinson, Franklin Lake, NJ). Hemoglobin was quantified before blood centrifugation for 5min at 3000g and 4°C. The pellet, corresponding to blood cells, was washed twice with 0.9% NaCl and frozen at –80°C until analyses. The supernatant, corresponding to the plasma, was sampled in aliquots and kept at –80°C until analyses. Total GSH in blood cells was determined using a diagnostic kit purchased by Calbiochem (EMD Biosciences Inc, Darmstadt, Germany) and is expressed in µmol GSH per ml of blood. The method is based on a colorimetric assay of a chromophoric thione formed specifically between the reagent and GSH. Total cysteine and cysteinyl-glycine were quantified in plasma with a technique adapted from(Jacobsen et al., 1994). Briefly, thiols were reduced and/or decoupled from proteins by reaction with Tris (2-carboxyethyl) phosphine(Krijt et al., 2001). After deproteinization with perchloric acid, thiols were derivatized with 7-fluorobenzofurazane-4-sulfonic acid (SBD-F). SBD-F derivates were analyzed by HPLC followed by fluorometric detection. Concentrations are expressed in µmol/l. From blood samples, analyses were primarily focused on GSH levels in blood as well as on levels of precursors: cysteine, one of the substrates of GCL, and cysteinyl-glycine, the product of GCL. These measures were assessed by a two-way, mixed effects ANOVA for crossover design with terms for Period (sequence), Patient and Treatment. The relevant effect of the treatment was adjusted for the random effect of the patient and for the study period (linked to the treatment sequence).

7.3 Results

7.3.1 Patients differ from controls at baseline

7.3.1.1 Behavioral results

Reaction times on the auditory discrimination task did not differ between patients (mean±SEM: 772±45ms) and healthy controls (610±77ms). Likewise, performance accuracy was near ceiling levels for both groups (patients: 98±1%; controls: 100±0%). This pattern of results thus argues against differences in selective attention or task performance as underlying AEP differences between groups.

7.3.1.2 Electrophysiological results

The topographic pattern analysis identified the same sequence of maps in both populations and thus provided no evidence for differences in the configuration of underlying brain networks either between conditions or populations. The results of that analysis will therefore not be discussed further. However, this method of defining AEP components provided a more objective means for defining time periods for the analysis of AEP and GFP waveforms. Likewise, the topography of the AEP (i.e. the location of peak amplitude) was used for the selection of specific scalp electrodes for the analysis of AEP waveforms. The N1 component of the AEP was identified over the 70-155ms interval.

AEP waveforms from four fronto-central electrodes are displayed in Figure 38a. Visual inspection of these waveforms indicates that healthy controls showed an early differential response (i.e. the MMN) between standard and deviant stimuli, whereas patients did not. This pattern is in keeping with what would be expected from prior investigations of the MMN in schizophrenia patients (Catts et al., 1995;Javitt et al., 1993;Javitt et al., 1998;Shelley et al., 1991;Shutara et al., 1996). To statistically assess whether our cohort of patients exhibited a deficient MMN, area measures (vs. the 0μV baseline) were taken from these electrodes over the 70-155ms interval and submitted to a 2x2x4 repeated measures ANOVA, using diagnosis for schizophrenia as the between subjects factor and stimulus condition and electrode as within subjects factors. Importantly, there was a significant interaction between diagnosis for schizophrenia and stimulus condition ($F_{(1,20)}$=11.605; p=0.003). Given this interaction, we then quantified the MMN for each population by calculating the average difference between responses to standard and deviant stimulus conditions across the 4 electrodes (bar graph in Figure 1a). These values were then submitted to a one-way ANOVA using diagnosis for schizophrenia as the between subjects factor. As expected, the MMN amplitude was significantly smaller in patients than in controls ($F_{(1,20)}$=11.626; p=0.003). An identical pattern of results was evident in the GFP waveforms (Figure 38b), which represent a global measure of the AEPs across the entire electrode montage and therefore are not influenced by a pre-selection of specific electrodes for analyses. GFP area measures (vs. the 0μV baseline) over the 70-155ms interval were submitted to a 2x2 repeated measures ANOVA, using diagnosis for schizophrenia as the between subjects factor and

stimulus condition as the within subjects factor. As above, there was a significant interaction between diagnosis for schizophrenia and stimulus condition ($F_{(1,20)}$=6.629; p=0.018). We then directly analyzed MMN amplitude with a one-way ANOVA, which revealed a significantly smaller MMN for patients than for controls ($F_{(1,20)}$=6.641; p=0.018; bar graphs in Figure 38b). Thus, analyses both at the level of single electrodes and also at the level of the global electric field strength confirmed that the MMN was deficient in our group of patients at protocol onset.

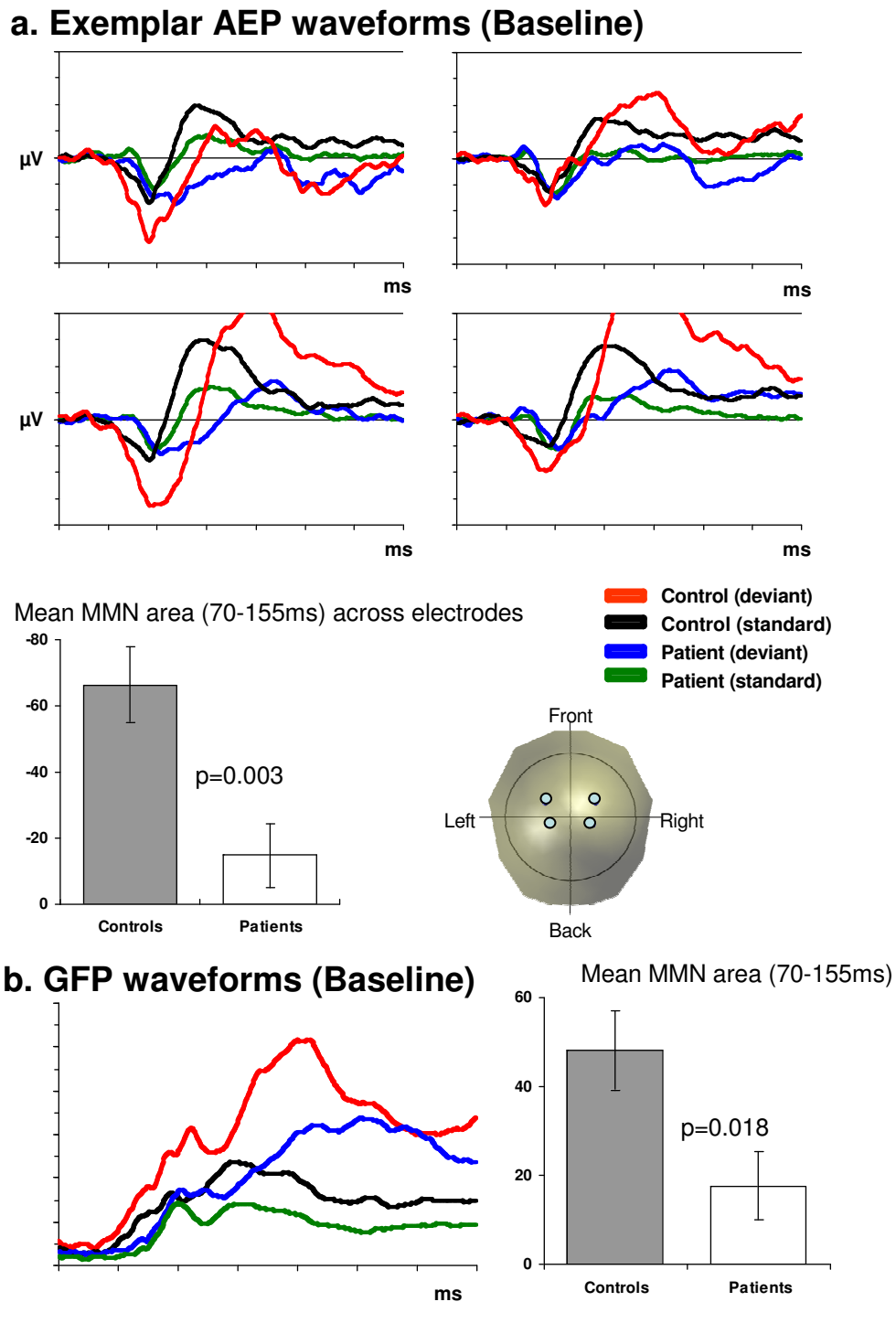


Figure 38. Electrophysiological responses at protocol onset from schizophrenia patients and healthy controls. *a*. The upper panels display exemplar AEP waveforms (voltage as a function of time) from a selection of fronto-central scalp locations (see inset for precise locations) in response to standard and deviant stimuli in both populations (color scheme indicated). Of particular interest is the initial negative-going component when a MMN is typically observed in healthy controls. The bar graph illustrates the group-averaged MMN (±SEM) during the 70-155ms post-stimulus period and averaged across the above scalp locations. *b*. GFP waveforms from all stimulus conditions and MMN magnitude are displayed for schizophrenia patients and healthy controls. Conventions are identical to those in *a*.

7.3.2 NAC increases MMN in schizophrenia patients

7.3.2.1 Behavioral results

Reaction times on the auditory discrimination task did not differ between patients following NAC (mean±SEM: 800±17ms) and placebo treatment (829±30ms). Likewise, performance accuracy was near ceiling levels for both treatments (NAC: 100±0%; placebo: 97±3%). This pattern of results thus argues against differences in selective attention or task performance as underlying AEP differences between treatments.

7.3.2.2 Electrophysiological results

As was the case at protocol onset, the topographic pattern analysis identified the same sequence of maps in response to both treatments and stimulus conditions. Thus, there was no evidence for differences in the configuration of underlying brain networks with NAC treatment or between stimulus conditions. The results of this analysis will therefore not be discussed further. The N1 component of the AEP was identified over the 70-155ms period, and the sequence of group-average AEP response topographies over the 80-120ms interval are displayed in Figure 39 for each treatment and stimulus condition. A fronto-central negativity was observed across both NAC and placebo treatments and both stimulus conditions. Inspection of the topography of the difference between stimulus conditions suggests that there was a robust MMN following NAC but not placebo treatment.

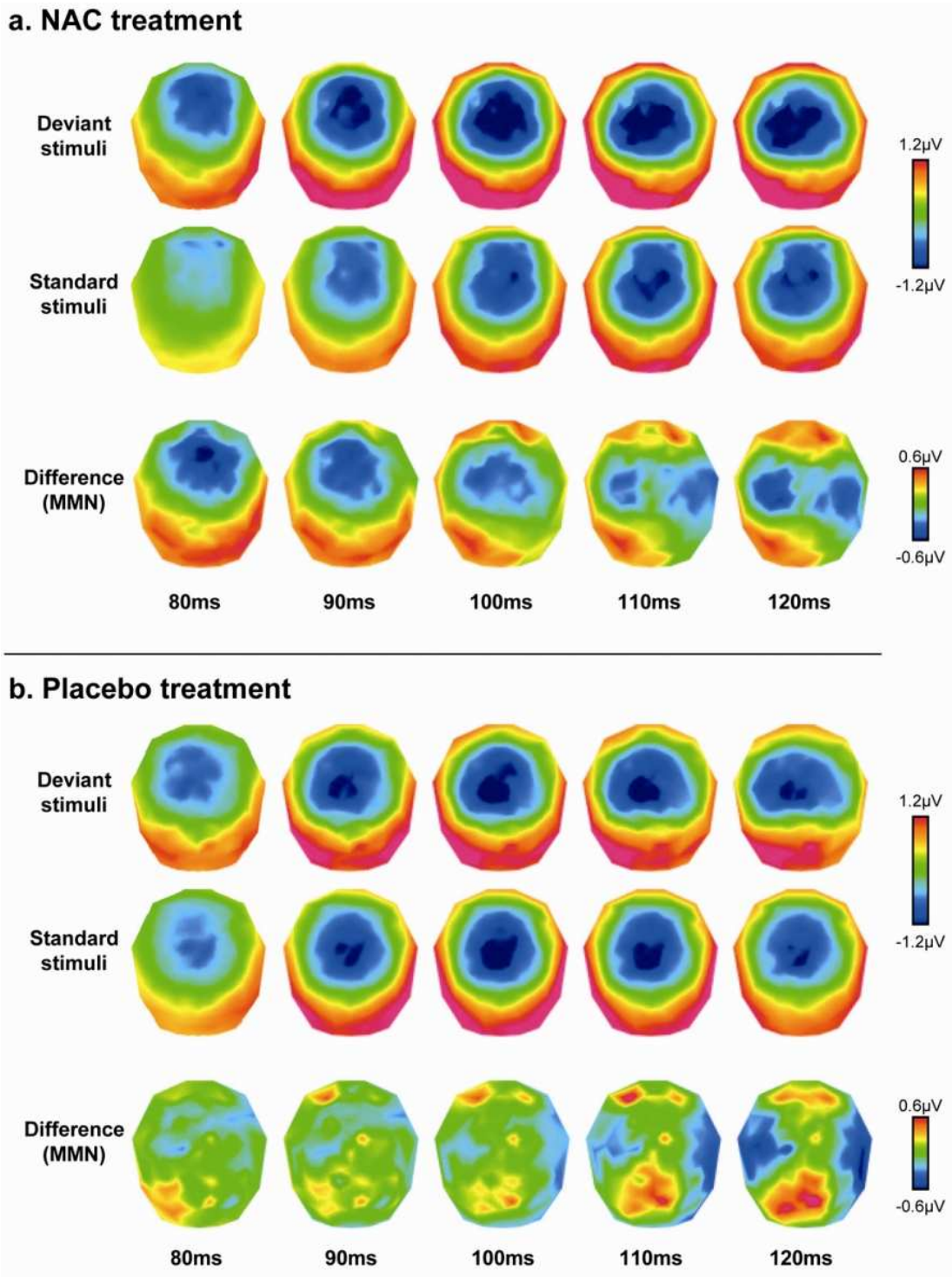


Figure 39. Topographic maps of AEP responses showing the distribution of voltage amplitude across the scalp at 10ms intervals from 80-120ms post-stimulus onset. Panels *a* and *b* show responses following NAC and placebo treatment, respectively, for each stimulus condition as well as their difference (i.e. the MMN).

AEP waveforms from four fronto-central electrodes are displayed in Figure 3a and suggest that N1 responses to deviant sounds were stronger following NAC than placebo treatment (red and blue traces, respectively), whereas responses to standard sounds did not differ (black and green traces, respectively). Area measures (vs. the 0μV baseline) were taken from these electrodes over the 70-155ms interval and submitted to a 2x2x4 repeated measures ANOVA, using treatment, stimulus condition, and electrode as the within subjects factors. The main effect of treatment did not reach the 0.05 significance criterion ($F_{(1,6)}$=0.739; p=0.423), providing no evidence for a global change in AEP amplitude with NAC treatment. There was a significant main effect of stimulus condition ($F_{(1,6)}$=21.206; p=0.004), following from the generally stronger responses to deviant than to standard stimulus conditions. Most critically, there was a significant interaction between treatment and stimulus condition ($F_{(1,6)}$=8.799; p=0.025; η_p^2=0.595). Given this interaction, we then quantified the MMN for each treatment by calculating the average difference between responses to standard and deviant stimulus conditions across the 4 electrodes (bar graph in Figure 3a). These values were then tested with a paired t-test and were significantly larger following NAC than placebo (-31.3±7.1μV vs. -7.5±4.2μV, respectively; $t_{(6)}$=2.966; p=0.025). An identical pattern of results was evident in the GFP waveforms (Figure 3b). GFP area measures (vs. the 0μV baseline) over the 70-155ms interval were submitted to a 2x2 repeated measures ANOVA, using treatment and stimulus condition as the within subjects factors. As above, there was a significant main effect of stimulus condition ($F_{(1,6)}$=20.562; p=0.004) and a significant interaction between treatment and stimulus condition ($F_{(1,6)}$=6.141; p<0.05; η_p^2=0.506). We then directly analyzed MMN amplitude with a paired t-test, which revealed a significantly larger MMN following NAC than placebo (27.1±6.1μV vs. 12.7±4.2μV, respectively, $t_{(6)}$=2.458; p<0.05; bar graphs in Figure 3b). Thus, analyses at the level of single electrodes and also at the level of the global electric field strength confirmed that the MMN was improved following NAC but not placebo treatment. We would note that all but one of the patients exhibited an enhanced MMN while receiving NAC relative to when receiving placebo treatment.

We also examined whether the later P300 component was modulated by whether or not patients were receiving NAC or placebo. For this analysis GFP area measures were calculated over the 235-600ms interval (see waveforms in Figure 3b), using the abovementioned topographic pattern analysis as a basis for the identification of AEP

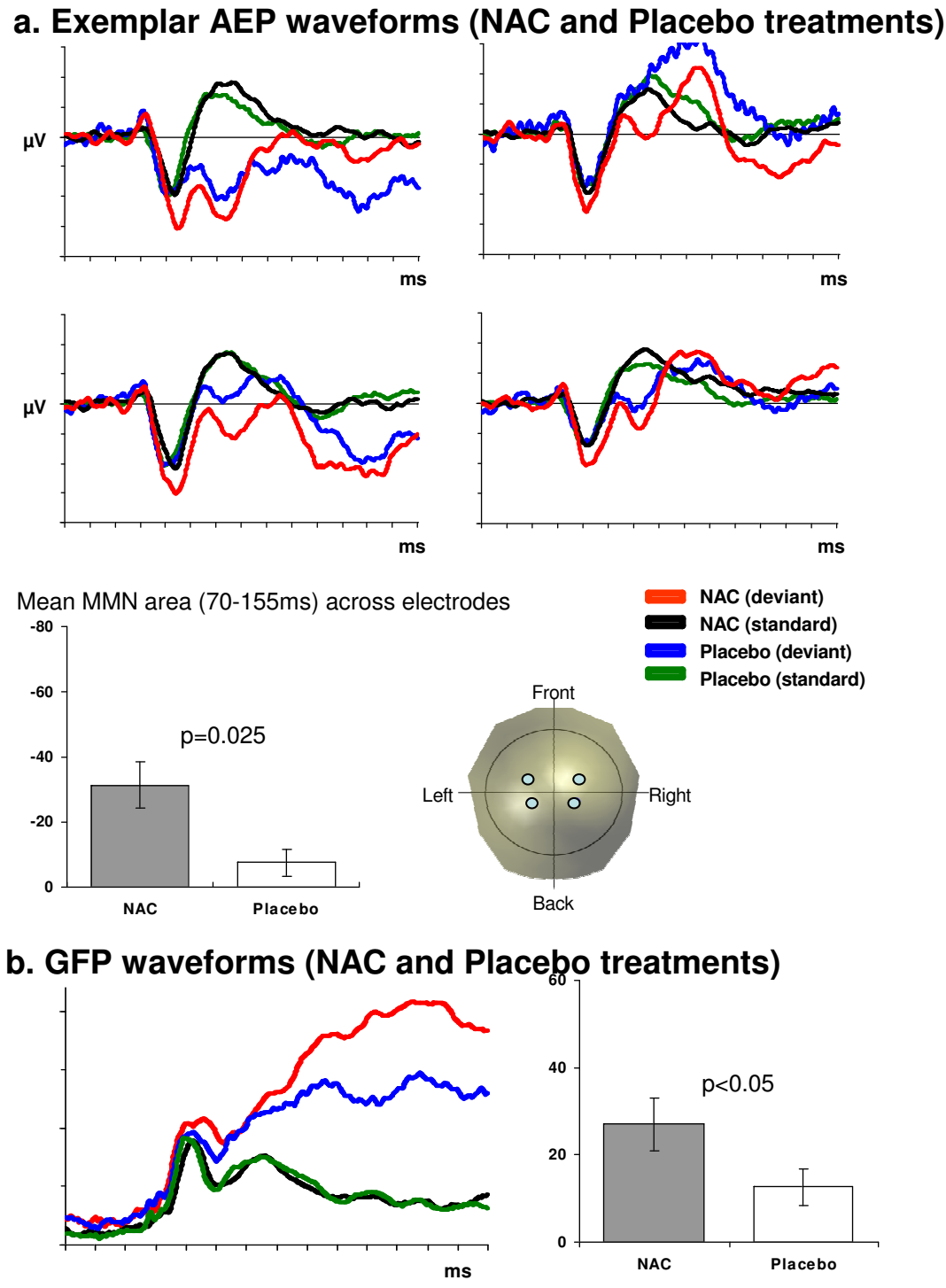


Figure 40. Electrophysiological responses following NAC and placebo treatment. *a*. The upper panels display exemplar AEP waveforms (voltage as a function of time) from a selection of fronto-central scalp locations (see inset for precise locations) in response to standard and deviant stimuli after each type of treatment (color scheme indicated). The bar graph illustrates the group-averaged MMN (±SEM) during the 70-155ms post-stimulus period and averaged across the above scalp locations. *b*. GFP waveforms from all stimulus conditions and MMN magnitude are displayed following each type of treatment. Conventions are identical to those in *a*.

components. These values were submitted to a 2x2 ANOVA using treatment and stimulus condition as within subjects factors. Only the main effect of stimulus condition was significant ($F_{(1,6)}$=30.212; p=0.002), whereas the main effect of treatment and the interaction between these factors both failed to meet the 0.05 significance criterion (all p-values>0.30). This pattern of results would suggest that the P300 was intact in patients both when receiving placebo as well as when receiving NAC. This AEP component showed no indication that it was modified by NAC treatment, in contrast to the earlier MMN response.

7.3.2.3 Neuropsychological and psychopathological results

The scores obtained during neuropsychological tests showed no effect of NAC treatment when compared to placebo (data not shown). These data were pooled with those of the larger clinical trial to be presented elsewhere (Berk et al., 2006).

7.3.2.4 Biochemical results

Levels of thiols linked to GSH were measured in blood from patients after NAC and after placebo treatment. Results are summarized in Table 2. After NAC treatment, an increase in total GSH levels was observed in whole blood ($F_{(1,9)}$=20.89; p=0.006) as well as in blood cells ($F_{(1,9)}$=29.87; p=0.001). However, this increase relative to placebo did not exceed 10%. There was no increase in cysteine and cysteinyl-glycine. No correlation was found between the magnitude of these biochemical changes and our electrophysiological measures.

	NAC treatment (mean ± SEM)	**Placebo** (mean ± SEM)	**ANOVA** *p*-value
GSH in blood cells (µmol/ml blood)	0.82 ± 0.05	0.76 ± 0.06	**0.001**
GSH in whole blood (µmol/ml blood)	0.89 ± 0.06	0.81 ± 0.07	**0.006**
Cysteine in plasma (µmol/l)	248.0 ± 10.0	262.4 ± 8.3	0.20
Cysteinyl-glycine in plasma (µmol/l)	43.1 ± 3.1	46.5 ± 7.3	0.17

Table 6. Levels of GSH and its cysteine-related precursors measured in blood from patients after NAC treatment and placebo. Bold *p*-values indicate significant difference.

8. Discussion

The present work has enabled us to demonstrate that it is possible to increase GSH levels using three different strategies: bypassing the limiting enzyme GCL, boosting the activity of this enzyme and providing more of the limiting precursor cysteine.

8.1 Bypassing the limiting enzyme GCL

8.1.1 Neurons *vs* astrocytes

In schizophrenia patients, the deficit in GSH observed is due to a defect at the level of the limiting synthesizing enzyme, GCL (Gysin et al., 2005;Tosic et al., 2006). We therefore used cultured astrocytes and neurons with a deficit in GSH as models. This deficit was induced with BSO, an inhibitor of GCL. To achieve the same percentage of decrease, that is about 70%, 10 times less BSO was necessary in astrocytes compared to neurons, indicating that GCL in astrocytes can be tuned more rapidly. Results from the group of Dalton suggest that, in most tissues, the GCLC/GCLM ratio is > 1, and that GCLM is limiting (Chen et al., 2005). It is possible that this ratio is lower in astrocytes than in neurons and, since BSO inhibits the catalytic subunit, a stronger inhibition will be nessary in neurons than in astrocytes to reach the same ratio. It is know that basal GCL activity in astrocytes is higher than in neurons (Gegg et al., 2003;Makar et al., 1994), which is in agrement with a low GCLC/GCLM ratio in astrocytes, considering that GCLM is limiting. Furthermore, astrocytes have been reported to have higher GSH levels than neurons (Bolanos et al., 1995;Gegg et al., 2003;Raps et al., 1989;Sagara et al., 1993). This difference in their GSH contents may account for their differential response to GCL inhibitors, such as BSO (Dukhande et al., 2006). Dukhande and collaborators also observed than less BSO is necessary to decrease GSH levels in astrcytes-like cells (U87) compared to neuron-like cells (SK-N-SH). In our hands, astrocytes presented GSH levels about three times higher than neurons, with basal values of 29.1 nmol/mg and 8.5 nmol/mg respectively.

8.1.2 GSHEE increases GSH levels in cultured astrocytes and neurons

BSO-treated cells allow us to investigate how an increase in GSH levels can be achieved when the limiting synthesizing enzyme is not functional. The only way to

reach our goal is by bypassing GCL. Since GSH itself is not transported inside the cell (Meister, 1989), membrane permeable esterified analogs of GSH and its precursor γ-glucys were used. 24-hour incubation with GSHEE significantly increased GSH contents in BSO-treated neurons and astrocytes to levels similar to those measured in control cells (Figures 11A and 12A). The concentration of GSHEE necessary to reach these levels was three times higher in astrocytes than in neurons, which is not surprising considering the basal GSH levels that are three times more elevated in astrocytes than in neurons. GSHEE also induced an increase in GSH contents in control neurons, but not in astrocytes, in which a plateau seems to be reached around the concentration of 30nmol/mg prot.

8.1.3 GCEE increases GSH levels in astrocyte, but not in neurons

GCEE could not increase GSH levels neither in controls, nor in BSO-treated neurons (Figure 13A), meaning that providing more γ-glucys is not leading to more GSH in this cell type. In view of these results, it seems like GSH analogs are more efficient than γ-glu-cys analogs in increasing GSH levels in neurons. These results contrast with those obtained by Boyd-Kimball and collaborators (2002), showing that GCEE can successfully increase GSH levels in BSO-treated neurons. This discrepancy is difficult to interpret since the duration of treatments, the concentration of GCEE, as well as the medium used, were the same in both their and our study. The only other difference is that they used hippocampal neurons from rats, while we used cortical neurons from mice. It has already been stated that the differences in the reported glutathione levels from a study to another might be attributed to differences in preparation techniques, species used, or culture conditions (Dringen, 2000). In astrocytes, GCEE was as much efficient as GSHEE, increasing GSH levels in BSO-treated cells to levels similar to those observed in controls (Figure 14A). A concentration of 3mM was again necessary, lower concentrations only partially, but linearly, increasing GSH levels. Our results suggest that the precursor γ-glucys is more readily used in astrocytes than in neurons. This is quite surprising, since it was shown that the extracellular concentration of glucys and γ-glucys providing half-maximal effects on GSH synthesis are lower in neurons than in astrocytes., indicating than neurons are more efficient in utilizing these compounds than astrocytes (Dringen, 2000). Furthermore, GSS, the enzyme that uses γ-glucys as a precursor, is usually not limiting. Howerver, lines of evidence suggest a

more prominent role for GSS in GSH regulation than previously recognized. For exemple, increased rat GSS and GCLC expression further enhanced GSH synthesis above that observed with increased GCLC expression alone (Huang et al., 2000).

8.1.4 GSH replenishment ameliorates NMDA function

In any case, GSHEE being more efficient than GCEE in increasing GSH levels in neurons, we chose to use this substance in the study of the effects of GSH replenishment in BSO-treated neurons on NMDA response. Recently, we observe that neurons with a deficit in GSH, show an altered modulation of NMDA responses by dopamine (Steullet et al., submitted). In control neurons, dopamine-enhanced NMDA-mediated calcium response (NMDA responses), while in BSO-treated neurons, dopamine decreased this response. Restoring normal GSH levels in BSO-treated neurons abolished the alteration of the dopamine modulation of NMDA responses (Figure 15). Indeed, the dopamine-induced decrease in NMDA responses in BSO-treated was no longer observed in BSO-treated neurons incubated with GSHEE. These results demonstrate that a depletion of intracellular GSH was responsible for the dopamine-induced decrease of NMDA responses observed in BSO-treated neurons and that GSH replenishment with GSHEE could re-establish normal dopamine modulation of these NMDA responses. Data suggest that enhancement of the function of redox sensitive ryanodine receptors (RyRs) in neurons with low GSH levels favors D2-type receptor-mediated and calcium-dependent pathways, causing a change in dopamine modulation of L-type calcium channels, and ultimately in dopamine modulation of NMDA response (Steullet et al., submitted). Rectifying the redox status with GSHEE in neurons probably abolishes this indirect effect of RyRs on NMDA response.

GSHEE being the direct analog of the substance we are interested in, that is GSH, it would be the best choice for a therapeutical approach using a substance that do not need a funtionnal GCL for increasing GSH levels. I.p.-injection or oral administration of GSHEE in mice increases GSH levels in liver and kidney (Anderson et al., 1985). Increase in these levels was also observed in spleen, pancreas, and heart, but not in brain. However, another study by the same group has shown that GSHEE is well transported into the cerebrospinal fluid (Anderson et al., 1989), indicating that GSH levels might be increased in only some parts of the brain.

More lipophilic derivates of GSH might also be useful. But, the toxicity of such derivative needs to be considered. Indeed, propanol, isopropanol and butanol are much more toxic than ethanol, the hydrolysis product of GSHEE (Anderson et al., 1985). GSH diethyl ester might be transported more rapidly than GSHEE (Levy et al., 1993). However, its deesterification is slower and the release of two ethanol instead one might represent a higher risk of toxicity. Morphological alterations with the ethyl esters of GSH has been noted previously (Scaduto, Jr. et al., 1988;Tsan et al., 1989), indicating that caution should be taken for its use *in vivo*. Considering the fact that GSH levels in the brain are in the range of the millimolar levels (Cooper and Kristal, 1997), a very high concentration of GSHEE could be necessary to reach these levels and toxicity could become an issue. In interesting alternative, the GSH-glycoside should be taken into consideration. Indeed, the effectiveness of GSH-glycoside in increasing GSH levels was demonstrated *in vitro* and *in vivo* (Choi et al., 1996). This substance is readily transported inside the cells and the glycoside release is unlikely to show toxicity.

8.2 Boosting the limiting enzyme GCL

BSO model was useful in the study of substances that bypass GCL. However, even if this enzyme is impaired in schizophrenia, its activity is unlikely to be completely blocked in patients. Therefore, boosting this enzyme in such a way that it produces more GSH is the second strategy that was used in the present thesis work. Based on literature, search for substances susceptible to increase GSH levels and GSH-related gene expression has led us to focus on curcumin, quercetin and tBHQ. Curcumin, a polyphenol, and quercetin, a flavonoid, are two naturally occurring compounds. TBHQ, a synthetic antioxidant, is a well-known activator of Nrf2 pathway. These three substances were shown to increase GSH levels in various cell types, most likely via an induction of phase II genes. In the present study, we were interested in increasing GSH levels in brain cells, and in studying the mechanisms by which this increase is achieved. We observed that curcumin and tBHQ could increase GSH levels in cultured neurons and astrocytes, while quercetin succeeded only in astrocytes.

8.2.1 Induction of phase II genes

All three substances were shown to induce phase II detoxifying and antioxidant-related genes (Lee et al., 2003;Myhrstad et al., 2002;Scapagnini et al., 2006;Vargas et al.,

2006). Curcumin, quercetin and tBHQ, although they have different chemical structures, all contain electrophilic unsaturated carbonyl groups. These groups can react selectively with nucleophiles such as thiols that are present on Keap1 (figure 5). The change in the conformation of keap-1 that is induced following the oxidation of the thiol group, leads to the dissociation of the cytoplasmic Nrf2-Keap1 complex, allowing Nrf2 to translocate into the nucleus (Jaiswal, 2004). The transcription factor Nrf2 was shown to be essential for the coordinate induction of phase II detoxifying and antioxidant enzymes, both of which are under ARE regulation (Ishii et al., 2000;Itoh et al., 1999). Nrf2 binds to the ARE and transcriptionally activates downstream target genes (Zhang and Gordon, 2004). Among these genes that contain an ARE sequence in their promoter, we find many GSH-related genes: Nrf2 itself (Kwak et al., 2002), glutathione-S-transferases (GST)(Rushmore and Pickett, 1990), the catalytic (Mulcahy et al., 1997) and the modulatory (Moinova and Mulcahy, 1998) subunits of GCL, as well as the specific subunit of the cysitine-glutamate exchanger, xCT (Sasaki et al., 2002). An ARE sequence was also described upstream of the promoter region of the gene of 4F2, the ubiquitous subunit of the exchanger (Sato et al., 2004), but its expression was not increased by diethyl maleate, an ARE inducer and thiol conjugate (Sasaki et al., 2002). Concerning GPx, the situation is not so obvious. Five GPxs were identified in mammals, all showing distinct roles in the cellular defense mechanisms. Gastrointestinal GPx gene was shown to be a target for Nrf2 (Brigelius-Flohe, 2006), but to date, no data are available about the action of Nrf2 towards brain GPx. Finally, GSS promoter region was recently characterized and two Nrf2 binding sites, highly homologous to ARE sequence, were identified (Lee et al., 2005).

Following treatment with curcumin, quercetin and tBHQ, we measured mRNA levels of Nrf2, of the two subunits of the cystine-glutamate exchanger (xCT and 4F2), the two subunits of the rate-limiting GSH synthesizing enzyme GCL [the modulatory (GCLM) and the catalytic (GCLC)], the second GSH synthesizing enzyme GSS and finally, GPx, one of the two enzymes implicated in the redox cycle. The second one, GST, being present in many isoforms, we did not measure their mRNA levels, but rather the activity of the enzymes using CDNB as a conjugate. This compound was shown to be the most ubiquitous in regard to the various isoforms of GST (Habig et al., 1974). Of course, being interested more specifically on boosting GCL, we measured the activity of this enzyme. Finally, in order to have an idea of the toxicity of the substances used towards

the cells, the activity of LDH leaking in the culture medium out of dying cells was measured.

8.2.2 Curcumin increases GSH levels in both astrocytes and neurons

Curcumin, a naturally occurring polyphenol, could increase GSH levels in neurons, and even more in astrocytes (Figures 21 and 22). Curcumin was shown to increase GSH in different cell lines, such as cultured human erythroleukemia (Singhal et al., 1999), type 1 astrocytes from rat (Scapagnini et al., 2002), human bronchial epithelial (Dickinson et al., 2003), human myelomonocytes (Strasser et al., 2005) and human alveolar epithelial (Biswas et al., 2005). This increase in GSH levels could be due to an increase of *de novo* GSH synthesis following the increased activity of GCL due to an induction of its expression by Nrf2. Indeed, curcumin was shown to increase Nrf2 protein levels in nuclear extracts from rat astrocytes, already one hour after the beginning of the treatment. Nrf2 translocation to the nucleus was accompanied by an upregulation of some phase II enzymes in these cells (Scapagnini et al., 2006).

Low concentration of curcumin (10µM) caused an increase in gene expression of Nrf2, xCT, 4F2 and GCLM in neurons, but only a small increase expression of GCLM and GSS in astrocytes. Thus, low curcumin concentration was able to boost more efficiently the whole system of GSH synthesis in neurons than in astrocytes. This certainly explains why such concentration of curcumin was more efficient in increasing GSH levels in neurons than in astrocytes. In neurons, the transport system for cystine was upregulated. This suggests that, although neurons in general seem to have a preference for the transport of cysteine (Shanker et al., 2001) via the excitatory amino acid transporter EAAT (Chen and Swanson, 2003), these cell can transport cystine when oxidative stress occurs. Even if only GCLM mRNA levels were increased, and not GCLC, the activity of the enzyme was increased by 50%, suggesting that upregulating the modulatory subunit is sufficient to boost GCL in neurons. Considering that GCLM is the limiting subunit (Chen et al., 2005), increasing its levels will lead to an increase in the GCLC/GCLM ratio, and therefore an increase in the catalytic activity of the enzyme.

In astrocytes, a higher concentration of curcumin (50µM), which led to a very high increased in GSH levels, strongly boosted the entire system of GSH synthesis, from the

cysteine uptake to the synthesis itself, apart from GPx. As mentioned earlier, brain GPx's promoter region has not been characterized yet (Brigelius-Flohe, 2006), and it is possible that it does not contain the ARE sequence. Furthermore, knowing that GPx is necessary for the reduction of ROS, free radicals might be better inducers than electrophiles. The gene expression of the two subunits of the cystine transporter, the system x_c^-, was upregulated, allowing more cysteine, the limiting precursor in the synthesis of GSH, to be available inside the cells. mRNA levels of the two GSH synthesizing enzymes were increased, with a really high level for the GCLM subunit. The increase in the gene expression of the two subunits of GCL was accompanied by an increase of 150% in the activity of the enzyme. However, with 100µM, while GCL activity is still high, the increase in GSH levels is less significant that with 50µM. This suggests that some of the GSH produced is immediately used.

The difference between the two cells types is not so surprising given that the expression of phase II detoxification enzymes is generally lower in neurons (Eftekharpour et al., 2000;Kraft et al., 2004;Murphy et al., 2001). Neurons can respond to lower concentrations, but they are unable to respond as strongly as astrocytes during a strong challenge.

Our results are consistant with those obtained using human lung epithelial cell lines. An increase in the content of *GCLC* (Biswas et al., 2005) and *GCLM* mRNA, as well as in the content of both GCL subunit proteins were observed after a treatment with curcumin (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). The decrease in GST activity that we observed in astrocytes are consistent with other studies showing that curcumin is a substrate inhibitor of human (Awasthi et al., 2000;Hayeshi et al., 2007) and rat (Oetari et al., 1996) GSTs. However, these results show a discrepancy with studies that demonstrated that curcumin could significantly increase the expression of GST, a member of the phase II detoxification enzymes in cultured astrocytes (Scapagnini et al., 2006). Furthermore, unsaturated carbonyl compounds are substrates for GSTs (van Iersel et al., 1997), therefore participating to their own elimination from the cell. *In vivo* studies will be required to assess the overall effect of curcumin compounds on GSTs. On the other hand, the observed increase in GSH indicates that formation of the conjugate of curcumin with GSH is not a significant route for

metabolism of curcumin, since this reaction would tend to decrease GSH levels. These results are consistent with previous metabolic studies in rat liver which indicated that curcumin is metabolized primarily to glucuronides and sulfates (Holder et al., 1978).

Our results show that curcumin is efficent in increasing GSH levels in both astrocytes and neurons. This substance could therefore be considered as a therapeutical approach for protecting cell against oxidative stress in the case of a deficit in GSH. Of course, apart from its ability to increase GSH levels, curcumin shows its own protective effect. Structurally, curcumin does not have the typical ring structure of polyphenol compounds, possessing a diketone group and two phenol rings that act as electron traps to prevent H_2O_2 production and to scavenge OH• and superoxide radicals. Besides, both the hydroxyl groups and the diketone moiety of curcumin are involved in metal-ligand complexation (Daniel et al., 2004). Curcumin can chelate Fe^{2-} needed for the generation of OH radicals (Reddy and Lokesh, 1994). However, at high concentration, this substance leads to a decrease in GSH levels accompanied by significant cell death in astrocytes. In neurons, neither GSH levels nor cell viability are decreased, but the trend in an increase in GSSG levels relative to GSH leads one to presume that higher concentrations could be noxious. Therefore, the choice of the concentration to be used in an *in vivo* would be primordial, considering that a concentration high enough to reach the brain will be necessary, but without inducing toxicity.

8.2.3 Quercetin increase GSH levels in astrocytes, but not in neurons

Quercetin, a naturally occurring flavonoid, could increase GSH levels in astrocytes, but not in neurons (Figures 24 and 25). This substance was shown to increase GSH levels in human breast cancer cells (Rodgers and Grant, 1998), monkey kidney derived cells (Myhrstad et al., 2002) and human hepatoma cell line (Alia et al., 2005;Scharf et al., 2003). In an *in vivo* study, quercetin could enhance the exploratory behavior, spatial learning and memory of the mice, these effects being related with an increase in brain GSH level (Liu et al., 2006). However, apart from its neuroprotective effects (Zhu et al., 2007), its effects in brain cells regarding GSH levels have never been investigated in cultures. This substance was shown to induce the ARE-dependent genes, in human breast carcinoma cell line (Valerio et al., 2001), and in monkey kidney cell line (Myhrstad et al., 2002).

In our study, quercetin induced roughly the same sets of GSH-related genes than curcumin in astrocytes. 20µM quercetin increased mRNA levels of xCT, GCLM and GSS in astrocytes already after 8h and, after 18h, the increase in GCLC gene expression reached significance. Quercetin seems to be less efficient that curcumin in inducing ARE-related genes and increasing GSH levels. This is consistant with a study that showed that curcumin, but not quercetin, is neuroprotective in a rat model of Parkinson's disease (Zbarsky et al., 2005). This result may also have been determined by variable metabolism by the intestinal flora and systemic availability and ability to cross the blood brain barrier (Manach and Donovan, 2004). For the cystine-glutamate exchanger, only the subunit xCT, but not 4F2, was induced by quercetin in astrocytes. xCT being specific for the cystine-glutamate exchanger, and 4F2 being ubiquitous to many transporters, it is possible that the expression of xCT is preferentially increased when the need for a boost in the cellular defense mechanisms occurs. As for curcumin, quercetin did not lead to an increase in mRNA levels of GPx and the two GSH synthesizing enzymes were upregulated, with a really high level for the GCLM subunit. The increase in the gene expression of the two subunits of GCL was accompanied by an increase of 100% in the activity of the enzyme. However, this increase led to only 50% more GSH. This result suggests that some of the *de novo* GSH synthesized is immediately used, either for the elimination of quercetin itself or for the scavenging of free radical potentially induced by the presence of quercetin in the cell. The last hypothesis does not seem realistic considering that the percentage of GSSG relative to GSH is unchanged. The polyphenolic structure of quercetin allows scavenging of free radicals, and when quercetin reacts with ROS, potentially harmful *ortho*-quinone (Boots et al., 2003). This product is then inactivated by GSH, to form two non reactive products: 6- glutathionyl-quercetin (6-GSQ) and 8-glutathionyl-quercetin (8-GSQ). In our study, no increase in the activity of GST was observed. Quercetin has been previously shown to inhibit GST (Kurata et al., 1992;van Zanden et al., 2003;Zhang and Das, 1994), but the nature of this inhibition has not been elucidated yet.

While no increase in cell death was observed in astrocytes, not only GSH levels were decreased in neurons, but quercetin, at high concentrations, led to an increase in cell death. Cell death after treatment with quercetin has been observed (Alia et al., 2005), and quercetin caused depletion of reduced glutathione which occurred prior to cell death (Duthie et al., 1997), probably because of the conjugation of the *ortho*-quinone with

GSH (Boots et al., 2003). Thus, it was proposed that quercetin can exert an antioxidant effect in the presence of high levels of GSH (Ferraresi et al., 2005), otherwise, it becomes pro-oxidants. Therefore, long exposure to quercetin results in a consumption of GSH and, in this case, reactive radical intermediates of quercetin cannot be scavenged anymore. As a consequence, the pro-oxidant effect of this molecule could prevail over the antioxidant effect. We cannot exclude that quercetin could boost GSH synthesis in neurons, but this could not compensate for the use of GSH for the elimination of quercetin itself. The pro-oxidant activity of quercetin may deplete the nuclear antioxidant defense, increasing nuclear lipid peroxidation and leading to oxidative DNA damage (Sahu and Gray, 1996). These results contrast with those showing that quercetin can protect membranes against lipid peroxidation (Decharneux et al., 1992;Laughton et al., 1991).

These contradictory results, but even more the fact that quercetin shows potential noxious effect, speak against the use of this substance in a therapy for increasing GSH levels.

8.2.4 tBHQ increases GSH levels in both astrocytes and neurons

tBHQ, a synthetic phenolic antioxidant, could increase GSH levels in both astrocytes, and neurons (Figures 27 and 28). tBHQ is a well studied inducer of phase II genes. This substance could strongly increase mRNA levels of all the GSH-related genes that we studied, except GPx in neurons and Nrf2 in astrocytes. The fact that the expression of Nrf2 was not increase in astrocyte is surprising considering the huge increase in mRNA levels of xCT and GCLM, already observed after 8h. In a study where tBHQ was used to induce ARE-driven genes in astrocytes, this substance did not increase Nrf2 expression levels, but induced nuclear translocation of Nrf2 (Lee et al., 2003). These results suggest that ARE activation by tBHQ is mediated by nuclear translocation of Nrf2, not by induction of Nrf2 gene expression in primary astrocytes. Furthermore, electrophilic compounds can induce these genes via the Keap1-Nrf2 pathway, but it seems like other pathways, one implying MAP kinase and another implying IP3 kinase, could also lead to the induction of ARE-driven genes (Nguyen et al., 2003). tBHQ is believed to be an activator of these cascades. This could explain why tBHQ activates ARE-regulated genes more strongly than curcumin and quercetin.

In our study, Nrf2 was induced in neurons, showing again the difference between the two cell types in the strategies they use to induce the antioxidant defense. Indeed, while neurons need to increase Nrf2 gene expression, astrocytes already containing enough of the protein can initiate the Nrf2/ARE cascade more rapidly. This could explain why the expression of some genes was already increased in astrocytes but not in neurons after only 8h. tBHQ could induced the expression of GSS in neurons, which was not the case for curcumin. tBHQ had stronger effects than curcumin on gene expression, but did not lead to larger GSL activity and GSH levels in neurons. This might be due to the limited increase in the gene expression of the catalytic subunit of GCL. This situation was also observed in astrocytes. Indeed, the 42 times increase in GCLM gene expression with 100µM tBHQ in astrocytes was accompanied by only 6.5 times increase in mRNA levels of GCLC. The activity of GCL was increased as much as after a treatment with curcumin, but not more. This suggests that even if GCLM is over expressed and positively modulates the activity of GCLC, if the catalytic subunit is not present in sufficient amount, the activity of the enzyme reaches a plateau. In this case the GCLC/GCLM ratio becomes inferior to 1. Like for quercetin treatment, the tBHQ-induced increase in GCL activity was higher than the increase in GSH levels. This suggests that some of the *the novo* GSH synthezised was immediately used, either for the elimination of tBHQ itself or for the scavenging of free radical potentially induced by the presence of tBHQ in the cell. Indeed, tBHQ, following a dealkylation step, can undergo oxidation-reduction reactions within cells and, in this case, is acting 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;Schilderman et al., 1993). These ROS must be detoxified and, indeed, the increase in the expression of GPx is accompanied by a trend in an increase in the percentage of GSSG relative to GSH with 100µM tBHQ. At this concentration, GSH levels are no longer significantly increased and a trend in cell death can be observed, meaning that the cell cannot cope anymore with the toxic effects of tBHQ. The same was observed in neurons, where 10µM and 20µM tBHQ could increase GSH levels, while higher concentrations led to cell death.

Despite tBHQ is a strong inducer of detoxifying and antioxydant enzymes, it potential toxic effect should be considered with caution, like it is the case of curcumin and quercetin at high concentrations.

8.2.5 Astrocytes are neuroprotectors

Our results, consistently with others, show that ARE-driven genes are prefentially activated in astrocytes compared to neurons (Eftekharpour et al., 2000;Kraft et al., 2004;Murphy et al., 2001), although at low concentrations of curcumin and tBHQ, neurons seems to be as efficiently, if not more, than astrocytes. Following stonger activation, astrocytes have more efficient GSH synthesis systems as well as higher GSH content than neurons, and export GSH to the extracellular media (Dringen et al., 2000). Secreted GSH can protect neurons acting as an antioxidant in the extracellular compartment and boosting GSH levels in neurons by increasing the availability of cysteine (Dringen et al., 1999).

8.2.6 The modulatory subunit is necessary for the action of the substances

Curcumin, quercetin and t-BHQ significantly increased the expression of the cystine-glutamate exchanger, the two subunits of GCL (GCLM and GCLC) and GSS in astrocytes of normal mice. This was accompanied by an increase in GCL activity and GSH levels. Because of the observed decrease in GCLM mRNA in fibroblasts of patients and the genetic association between the GCLM gene and schizophrenia, we assessed the importance of this subunit in the induction of GCL and the subsequent increase in GSH levels, using astrocytes from GCLM KO mice. The three substances could significantly increase GCLC gene expression in astrocytes from normal mice (OF1). Due to material limitation, mRNA levels could not be measured in cells from GCLM KO mice. We suppose that GCLC gene regulation is the same in astrocytes from OF1 and from GCLM KO. The inhability of the substances to increase GCL activity and GSH levels in astrocytes lacking a functional GCLM demonstrates that GCLM was necessary to increase GSH levels. An upregulation of cystine transport, GCLC subunit and GSS was not sufficient in absence of GCLM. However, even if a defect in GCLM is related to schizophrenia, patients are not knocked-out for this gene, so upregulating its expression could theoretically still be achieved.

8.2.7 FK506 has no effect in both astrocytes and neurons

Finally, we also tested the efficacy of an immunosuppressant, FK 506, in increasing GSH levels in cultured neurons and astrocytes. Indeed, the group of Tanaka *et al.* (2000,

2001, 2002 and 2004), showed that GSH levels were increase by FK506 in neuroblastoma cell line, neuroblastoma-glioma hybrid cell line and glioma cell lines. However, the mechanisms by which this immunosuppressant can increase GSH levels are unclear. The same group has conducted an *in vivo* study during which FK506 was i.p.-injected to mice and they observed an increase in GSH levels in striatum, as well as an increase in gene expression of GCL. However, no distinction was done between the whole GCL and its subunits, regarding the mRNA quantification, which it quite intriguing. No change in mRNA levels of GPx or GST were observed, leading one to doubt about the phase II enzyme-induction activity of FK506. In our hand, FK506 had no effect on GSH levels in both neurons and astrocytes, neither did the cell viability was affected. Therefore, no further investigations were done.

8.3 Providing more of the limiting precursor cysteine

NAC, a precursor of GSH, given as an add-on treatment (2 g / day for 2 months) to schizophrenia patients, induces an increase in auditory evoked MMN, likely reflecting an improvement in NMDA-R function.

8.3.1 MMN is impaired in schizophrenia patients

It had already been shown that MMN is impaired in schizophrenia patients (Catts et al., 1995;Javitt et al., 1993;Javitt et al., 1998;Shelley et al., 1991;Shutara et al., 1996) and this was the case again in our small group of patients at protocol onset, compared to sex and age-matched controls (figure 38). In fact, during the auditory oddball paradigm (AOP), the difference observed in controls between the GFP induced by the target and by the distractor around 100 ms was not present in patients at the beginnig of the clinical trial. GFP is equivalent to the spatial standard deviation of the scalp electric field, and quantifies the amount of activity at each time point in the field, considering the data from all recording electrodes simultaneously. It is therefore a measure of the global strength of the scalp electric field at each time-point and these values are plotted as a function of time. Changes in the amplitude of the GFP indicate a change in the strength of the underlying generator, while a change in the map topography indicates a change in the generators themselves. The map topography induced by the target was the same for control and patients between 70 and 155 ms (figure 39). However, the

amplitude of the GFP was different, indicating that the capacity to evoke MMN is reduced in schizophrenia patients.

8.3.2 NAC improves MMN generation

A group of patients was treated with NAC for two months and then with placebo for another two-month period, and vice-versa for a second group. Our results show that patients treated with 2g of NAC per day show improved MMN. Analyses of AEPs recorded after NAC treatment versus placebo revealed the efficacy of this GSH precursor in modulating MMN generation mechanisms. In fact, we observed an increase in the amplitude of the GFP of MMN after NAC treatment that was not present after placebo (figure 40), indicating that NAC ameliorates MMN generation.

8.3.4 NAC increases blood GSH levels

An increase of GSH levels in blood was measured in our subset of patients after NAC treatment (table 6), showing that NAC was absorbed, at least in part, by the gastro-intestinal system. No increase in cysteine levels was observed in blood from patients after NAC treatment. This could suggest that all the deacetylated cysteine is immediately used and that higher doses of NAC could be considered for treatment. On the other hand, even though single oral doses of NAC leads to an increase in NAC levels in plasma, it does not appear to accumulate (Borgstrom and Kagedal, 1990). In this case, a more frequent intake of NAC would ensure more sustained concentrations.

The present study was conducted in the context of a multicenter clinical trial. The effectiveness of NAC in reducing clinical severity was demonstrated over the whole set of 140 patients included in the multicenter trial and randomly assigned to either NAC or placebo treatment (Berk et al., 2006). An improvement on the Clinical Global Impression (CGI) was observed already two weeks after the beginning of the trial and was sustained for the 6-months trial period. An improvement of symptoms on the Positive and Negative Symptoms Scale (PANSS) was also significant after 6 months of treatment with NAC.

We hypothesise that a fraction of the oral NAC entered blood flow, crossed the blood-brain barrier (Farr et al., 2003), was deacetylated into cysteine that could readily be used to increase GSH levels. As already mentioned, GSH being the major antioxidant in a

cell system, its presence is necessary to maintain equilibrium on the redox status. In fact, redox-sensitive proteins such as NMDA-R, which are implicated in the generation of MMN (Javitt et al., 1996), can have their activity decreased when the ratio GSH/GSSG becomes too low (Janaky et al., 1993;Sucher and Lipton, 1991). Indeed, GSH deficit in hippocampus slices leads to NMDA-R hypofunction and inhibition of long-term potentiation (Steullet et al., 2006). Therefore, it is plausible that the absence of MMN in patients could be due, at least in part, to hypofunction of NMDA-R subsequent to a GSH deficit. In patients with low brain GSH, NAC could increase GSH levels, and thus improving the functioning of NMDA-R. Therefore, the increase of MMN observed after NAC treatment is likely to correlate with a better functioning of NMDA-R.

However, from our data, we cannot conclude with certitude that the effect of NAC is via its cysteine donor property. In fact, NAC being an antioxidant itself, it is possible that it has a direct effect on the redox properties of NMDA-R. NAC can also affect gene regulation of detoxifying enzymes by activating redox-sensitive transcription factors, such as nuclear factor κB (NF-κB) and activator protein 1 (AP-1; Cotgreave, 1997). To assess if NAC really elevates GSH levels in the brain of schizophrenia patients, *in vivo* magnetic resonance spectroscopy (MRS) would be necessary. However, it has been reported that with current MRS methods, the signal to noise ratio would need to be improved in order to observe discrete increases in brain GSH levels (Trabesinger et al., 1999).

9. Conclusion

GSHEE, a substance that can penetrate the cells, be de-esterified and directly be used as GSH in the cell, is highly efficient in increasing GSH levels and showed no toxicity in our models. Using this subtance in neurons previously deprived in GSH with BSO, could restore normal NMDA functions, that was impaired in the presence of dopamine. This substance is therefore of high interest in a therapy aiming at increasing GSH levels, since its action is direct and, at first glance, safe. However, considering the high concentrations that would be necessary for the substance to reach the brain and efficiently increase GSH levels inside brain cells, finding the appropriate delivery mode remains a challenge.

Substances that can induce ARE-driven genes, including the two subunit of GCL, the limiting enzyme in the synthesis of GSH, are tempting therapeutic agents. Indeed, curcumin and tBHQ could increase GSH levels in both neurons and astrocytes, and quercetin was efficient in astrocytes. Their potential toxicity leads one to be cautious before considering it as a clinical treatment. However, considering the low bioavailability of these substances, it would be surprising that toxic levels would be reached in the brain.

MMN, a component of the AEP that is typically impaired in schizophrenia, was improved following a treatment with NAC, a precursor of GSH, supporting the hypothesis of a deficit in GSH in the brain of schizophrenia patients. However, considering that brain GSH deficiency in schizophrenia seems to be due to a defect in the key synthesizing enzyme GCL (Tosic et al., 2006), a cysteine precursor might not represent the best substance to use in order to increase GSH levels. Substances that bypass the enzyme GCL or that boost this enzyme, as discussed in the two previous sections would represent better choices, however raising new challenges for their efficient delivery into the brain.

In conclusion, modulating GSH levels in the brain of patients might already be considered as a promising pathway towards an effective long-term therapeutic strategy in schizophrenia. Indeed, in view of the side effects induced by current neuroleptics, clinicians should consider the administration of naturally occurring antioxidants, or safe GSH analogs or precursors. These substances are interesting for high-risk patients during prodromic phase or first episode patients with a diagnosis not well established.

10. Abbreviations

ARE	antioxidant response element
BSO	L-buthionine-(S,R)-sulfoximine
GCEE	γ-glutamylcysteine ethyl ester
GCL	glutamylcysteine ligase
GCLM	glutamylcysteine ligase modulatory subunit
GCLC	glutamylcysteine ligase catalytic subunit
GFP	global field power
γ-glucys	γ-glutamylcysteine
GPx	glutathione peroxidase
GSH	glutathione
GSHEE	glutathione ethyl ester
GSR	glutathione reductase
GSS	glutathion synthetase
GST	GSH-S-transferase
γGT	γ-glutamyl transpeptidase
HZ	heterozygote
KO	knock-out
MMN	mismatch negativity
NAC	N-acetyl-cysteine
ROS	reactive oxygen species
WT	wild-type

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